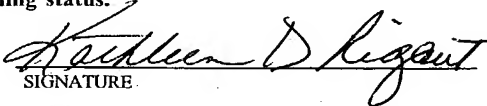


FORM PTO-1390 (REV. 12-2001)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER	
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371				RUT.00-0038US	
				U.S. APPLICATION NO. (If known, see 37 CFR 1.5 not yet assigned) 10/088634	
INTERNATIONAL APPLICATION NO. PCT/US00/25939		INTERNATIONAL FILING DATE 21 September 2000		PRIORITY DATE CLAIMED 21 September 1999	
TITLE OF INVENTION SITE-SPECIFIC RECOMBINATION SYSTEM TO MANIPULATE THE PLASTID GENOME OF HIGHER PLANTS					
APPLICANT(S) FOR DO/EO/US MALIGA, Pal; CORNEILLE, Sylvie; LUTZ, Kerry					
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:					
<p>1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.</p> <p>2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.</p> <p>3. <input checked="" type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.</p> <p>4. <input checked="" type="checkbox"/> The US has been elected by the expiration of 19 months from the priority date (Article 31).</p> <p>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))</p> <p style="margin-left: 20px;">a. <input type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau).</p> <p style="margin-left: 20px;">b. <input checked="" type="checkbox"/> has been communicated by the International Bureau.</p> <p style="margin-left: 20px;">c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</p> <p>6. <input type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).</p> <p style="margin-left: 20px;">a. <input type="checkbox"/> is attached hereto.</p> <p style="margin-left: 20px;">b. <input type="checkbox"/> has been previously submitted under 35 U.S.C. 154(d)(4).</p> <p>7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))</p> <p style="margin-left: 20px;">a. <input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau).</p> <p style="margin-left: 20px;">b. <input type="checkbox"/> have been communicated by the International Bureau.</p> <p style="margin-left: 20px;">c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</p> <p style="margin-left: 20px;">d. <input type="checkbox"/> have not been made and will not be made.</p> <p>8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).</p> <p>9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).</p> <p>10. <input type="checkbox"/> An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</p> <p>Items 11 to 20 below concern document(s) or information included:</p> <p>11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</p> <p>12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</p> <p>13. <input checked="" type="checkbox"/> A FIRST preliminary amendment.</p> <p>14. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.</p> <p>15. <input type="checkbox"/> A substitute specification.</p> <p>16. <input type="checkbox"/> A change of power of attorney and/or address letter.</p> <p>17. <input type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.</p> <p>18. <input type="checkbox"/> A second copy of the published international application under 35 U.S.C. 154(d)(4).</p> <p>19. <input type="checkbox"/> A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).</p> <p>20. <input checked="" type="checkbox"/> Other items or information: Copy of Form PCT/IB/308</p>					

U.S. APPLICATION NO. (if known, see 37 CFR 1.51) Int / ver 88634		INTERNATIONAL APPLICATION NO. PCT/US00/25930		ATTORNEY'S DOCKET NUMBER RUT.00-0038US	
21. <input checked="" type="checkbox"/> The following fees are submitted:				CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):					
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO.....				\$1040.00	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO				\$890.00	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO				\$740.00	
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4)				\$710.00	
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4)				\$100.00	
ENTER APPROPRIATE BASIC FEE AMOUNT =				\$ 100.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	\$	
Total claims	21 - 20 =	1	x \$18.00	\$ 18.00	
Independent claims	2 - 3 =	0	x \$84.00	\$ 0	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$280.00	\$ 0	
TOTAL OF ABOVE CALCULATIONS =				\$ 118.00	
<input checked="" type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				\$ 59.00	
SUBTOTAL =				\$ 59.00	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				\$ 0	
TOTAL NATIONAL FEE =				\$ 59.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +				\$ 0	
TOTAL FEES ENCLOSED =				\$ 59.00	
				Amount to be refunded:	\$
				charged:	\$
a. <input checked="" type="checkbox"/> A check in the amount of \$ <u>59.00</u> to cover the above fees is enclosed.					
b. <input type="checkbox"/> Please charge my Deposit Account No. _____ in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.					
c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>04-1406</u> . A duplicate copy of this sheet is enclosed.					
d. <input type="checkbox"/> Fees are to be charged to a credit card. WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.					
NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.					
SEND ALL CORRESPONDENCE TO:					
Kathleen D. Rigaut, Ph.D., J.D. DANN, DORFMAN, HERRELL AND SKILLMAN 1601 Market Street, Suite 720 Philadelphia, Pennsylvania 19103 United States of America					
				 SIGNATURE	
				Kathleen D. Rigaut, Ph.D., J.D. NAME	
				<u>43,047</u> REGISTRATION NUMBER	

JC13 Rec'd PCT/PTO 20 MAR 2002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of)
)
Pal Maliga et al.)
)
Serial No. Not yet assigned)
[Int. Appl. No. PCT/US00/25930])
)
Filed: September 21, 2000)
)
For: "Site-specific)
Recombination System to)
Manipulate the Plastid)
Genome of Higher Plants")

PRELIMINARY AMENDMENT

The present application is based on International Application PCT/US00/25930. Before calculation of the filing fee, please amend the above-referenced patent application as follows:

In the specification:

At page 1, line 5 of the specification, please insert the following priority claim:

-- This application is a §371 filing of PCT/US00/25930 filed September 21, 2000 which in turn claims priority to US provisional applications 60/155,007 and 60/211,139 filed September 21, 1999 and June 13, 2000, respectively, each of which is incorporated by reference herein.--

At page 60 of the application, on a separate sheet, please insert a copy of the abstract which attached hereto.

REMARKS

The purpose of this preliminary amendment is insert a priority claim into the application and to provide a copy of the abstract on a separate sheet.

Favorable consideration leading to prompt allowance of the present application is respectfully requested.

Respectfully submitted,
DANN, DORFMAN, HERRELL AND SKILLMAN
A Professional Corporation

By 
Kathleen D. Rigaut, Ph.D., J.D.
PTO Registration No. 43,047

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ABSTRACT

A site-specific recombination system and methods of use thereof are disclosed for manipulating the genome of higher plants.

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PCT/US00/25930

Rec'd PCT/PTO 20 MAR 2002

Site-specific recombination system to manipulate the
plastid genome of higher plants

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10 This application claims priority under 35 U.S.C.
§119(e) to US Provisional Applications 60/155,007 and
60/211,139 filed September 21, 1999 and June 13, 2000
respectively, the entire disclosure of each of the
above-identified applications is incorporated by
reference herein.

15

FIELD OF THE INVENTION

20 This invention relates to the fields of transgenic
plants and molecular biology. More specifically, DNA
constructs and methods of use thereof are provided which
facilitate the excision of target DNA sequences from
transplastomic plants.

BACKGROUND OF THE INVENTION

25 Several publications are referenced in this
application by author name and year of publication in
parentheses in order to more fully describe the state of
the art to which this invention pertains. Full
citations for these reference can be found at the end of
30 the specification. The disclosure of each of these
publications is incorporated by reference herein.

35 The plastid genetic system of higher plants is
highly polyploid. For example, in a tobacco leaf there
are as many as 100 chloroplasts, each carrying ~100
identical genome copies, a total of 10,000 copies in a
leaf cell. High-level protein expression, lack of
pollen transmission and the feasibility to engineer
polycistronic expression units make the plastid genome

an attractive alternative to nuclear engineering.

Plastid transformation vectors often contain a selective marker, most commonly a spectinomycin resistance (*aadA*)

gene, flanked by plastid DNA sequences targeting

5 insertion of the marker gene by homologous recombination into the plastid genome. Genes of commercial value but

lacking a selectable phenotype are physically linked to the selective marker and the two genes are integrated

together as a block of heterologous sequences. Plastid

10 transformation is accomplished by biolistic DNA delivery or polyethylene glycol induced uptake of the transforming DNA followed by selection for the

antibiotic resistance marker to ensure preferential propagation of plastids with transformed genome copies.

15 As the result, all the 10,000 wild-type plastid genome copies in a cell are replaced with transgenic copies during a gradual process (Maliga, 1993).

Incorporation of a selectable marker gene is essential to ensure preferential maintenance of the transformed plastid genome copies. However, once

20 transformation is accomplished, maintenance of the marker gene is undesirable. One problem may be the metabolic burden imposed by the expression of the selectable marker gene. For example FLARE-S, the product

25 of the marker gene with good prospects to transform cereal chloroplasts, accumulates up to 18% of the total soluble cellular protein (Khan and Maliga 1999). The second problem is the relatively high potential for horizontal transfer of plastid marker genes to microbes

30 (Tepfer 1989; Dröge et al. 1998; Sylvanen 1999), as commonly used plastid marker gene constructs are efficiently expressed in *E. coli* (Carrer et al. 1993; Svab and Maliga 1993). Therefore, having plastid marker genes in commercial products is undesirable.

SUMMARY OF THE INVENTION

In accordance with the present invention, methods and systems are provided which facilitate the manipulation of the plastid genomes of higher plants. The methods and systems of the invention may be employed to remove heterologous sequences from the plastid genome, such as selectable marker genes following successful isolation of transformed progeny. Alternatively, they may be designed to remove endogenous genes involved in plant cell metabolism, growth, development and fertility.

In one embodiment of the invention, a site specific recombination method for removal of predetermined nucleic acid sequences from the plastid genome is provided. The method comprises providing a first nucleic acid construct, the construct comprising a promoter being operably linked to a nucleic acid encoding an optional plastid targeting transit sequence which is in turn operably linked to a nucleic acid encoding a protein having excision activity, the construct further comprising a first selectable marker encoding nucleic acid having plant specific 5' and 3' regulatory nucleic acid sequences. The method also entails the use of a second DNA construct, the second construct comprising an second selectable marker encoding nucleic acid and excision sites. The second construct optionally contains a gene of interest and further comprises flanking plastid targeting nucleic acid sequences which facilitate homologous recombination into said plastid genome. The second DNA construct is introduced into plant cell and the cells are cultured in the presence of a selection agent, thereby selecting for those plant cells expressing the proteins encoded by said second DNA construct. The first DNA construct is

then introduced into cells having the second construct in the presence of a selection agent and those plant cells expressing proteins encoded by said first construct are selected. If present, the excising activity acts on the excision sites, thereby excising said predetermined target sequence. Plants may then be regenerated from plant cells obtained by the foregoing method.

Proteins having excision activity suitable for the practice of the invention include, without limitation, CRE, flippase, resolvase, FLP, SSV1-encoded integrase, and transposase. Sequences corresponding to excision sites suitable for the practice of the invention, include, for example, LOX sequences, and *frt* sequences.

A variety of selection of agents may be selected. These include without limitation, kanamycin, gentamycin, spectinomycin, streptomycin and hygromycin, phosphinotricin, basta, glyphosate and bromoxynil.

In an alternative embodiment, a site specific recombination method for removal of predetermined nucleic acid sequences from the plastid genome is provided. The method comprising providing a first nucleic acid construct, said construct comprising a regulated promoter being operably linked to a nucleic acid encoding an optional plastid targeting transit sequence which is operably linked to a nucleic acid encoding a protein having excision activity, said construct optionally further comprising a first selectable marker encoding nucleic acid having plant specific 5' and 3' regulatory nucleic acid sequences. A second DNA construct is also provided, said second construct comprising an second selectable marker encoding nucleic acid and excision sites, said second construct further comprising flanking plastid targeting nucleic acid sequences which facilitate homologous

recombination into said plastid genome at a predetermined target sequence such that excision sites flank said predetermined target sequence following homologous recombination and introducing said second DNA construct into a plant cell. The plant cell so generated is then cultured in the presence of a selection agent, thereby selecting for those plant cells expressing the proteins encoded by said second DNA construct. A plant is then regenerated from cells containing the second construct and the first DNA construct is introduced into these cells in the presence of a selection agent and those plant cells expressing proteins encoded by said first construct are selected. The excising activity then acts on the excision sites, thereby excising said predetermined target sequence.

Regulatable promoters suitable for this embodiment of the invention include, without limitation, inducible promoters, tissue specific promoters, developmentally regulated promoters and chemically inducible promoters.

Candidate predetermined target sequences, may include for example genes associated with male sterility, *clpP*, ribosomal proteins, ribosomal operon sequences.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram depicting CRE-mediated excision and integration of DNA segments.

Figure 2 is a map of a plastid transformation vector pSAC48, with *codA* bracketed by direct *loxP* sites. Positions of plastid genes *rrn16*, *trnV*, *rps12/7* (Shinozaki et al. 1986), the *aadA* and *codA* transgenes and relevant restriction sites are marked.

Figure 3 is a map of an *Agrobacterium* binary vector

pPZP212 with a plastid-targeted *Ssu-tp-cre* gene. Marked are: *Agrobacterium* Left and Right Border fragments; the kanamycin resistance (*neo*) gene; P2' promoter; SSU transit peptide (*ssu-tp*); *cre* coding region; recognition sequences for restriction enzymes *Bam*HI, *Eco*RI, *Hind*III, *Nco*I, *Nhe*I and *Xba*I.

Figure 4 shows maps of the plastid genome *>codA* deletion derivatives. Shown are the plastid targeting region of vector pSAC48; the map of same region of the wild-type plastid genome (Nt-wt); the map of the plastid genome with CRE-mediated deletion of *codA* via the *lox* sites; and the map of the plastid genome with deletion via *Prrn* sequences lacking *trnV*, *aadA* and *codA*.

Positions of plastid genes *rrn16*, *trnV* and *rps12/7* (Shinozaki et al. 1986), *aadA* and *codA* transgenes, primers (O1-O4) and relevant restriction sites (*A*I, *Apa*I; *E*V, *Eco*RV) are marked.

Figure 5 is a gel showing PCR amplification which confirms CRE-mediated deletion of *codA* from the plastid genome. Primers O1 and O2 (Fig. 3) amplified the 0.7-kb fragment of the deleted region. Same primers amplify the 2.0-kb *aadA-codA* fragment in tester lines Nt-pSAC48-21A and Nt-pSAC-16C (no transgenic Cre gene). No specific fragment was obtained in wild-type DNA sample and in Cre1-10 line. The lines obtained are listed in Table 1.

Figure 6 shows the results of DNA gel blot analysis wherein plastid genome structure was determined in the indicated plant samples. Total cellular DNA was isolated from the leaves of plants listed in Table 1 and digested with the *Apa*I and *Eco*RV restriction endonucleases. The probes were the wild-type *Apa*I-*Eco*RV plastid targeting

region and the *aadA* (*NcoI*-*XbaI* fragment) and *codA* (*NcoI*-*XbaI* fragment) coding regions. The hybridizing fragments are marked in Fig. 3.

5 Figure 7 are gels showing uniformity of plastid genome populations in the *Ssu-tp-cre* transformed plants. Total cellular DNA extracted from several leaves was probed with the *ApaI-EcoRV* targeting region probe. Numbers identify leaves from which DNA was extracted. 10 For example, seven different leaves were probed from the Cre1-3 plant. For details, see Brief Description of Fig. 6.

15 Figures 8A and 8B are gels of PCR analysis confirming CRE-mediated deletion of *codA* in seedlings obtained by pollination with *Ssu-tp-cre* activator lines. 5-day old seedlings were tested from the cross Nt-pSAC48-21A as maternal parent and Cre2-200 and Cre2-300 activator lines as pollen parents. Amplification 20 products are also shown for controls Nt-pSAC48-21A selfed seedling (48 self), wild-type (wt), the parental plant (48P) and the Cre1-3 plant. Fig. 8A: The *codA* region was amplified with the 01/02 primers: the size of *aadA-codA* fragment is 2.0 kb; the *codA* deletion fragment 25 is 0.7 kb (Fig. 4). Fig. 8B: Testing for *cre* sequences by PCR amplification with the Cre1/Cre3 oligonucleotides.

30 Figure 9 is a diagram of the plastid transformation pSAC38 with the >neo< bracketed by inverted lox sites. Positions of plastid genes *rrn16*, *trnV* and *rps12/7* (Shinozaki et al., 1986), the *aadA* and *codA* transgenes and relevant restriction sites are marked.

35 Figure 10 shows a map of the plastid genome

containing the >neo< inversion construct. Shown are the plastid targeting region of vector pSAC38; the map of the same region of the wild-type plastid genome (Nt-wt); map of the plastid genome with CRE-mediated inversion of neo via the lox sites. Positions of the plastid genes *rrn16*, *trnV* and *rps12/7* (Shinozaki et al., 1986) *aadA* and neo transgenes, primers (O1-O4) and relevant restriction sites (BamHI) are marked.

Figure 11 shows the results of DNA gel blot analysis for the determination of plastid genome structure of CRE-activated >neo< plants by DNA gel blot analysis. Total cellular DNA was digested with the BamHI restriction endonuclease. The probes was the wild-type *ApaI-EcoRV* plastid targeting region. The hybridizing fragments are marked in Fig. 10.

Figure 12 shows an exemplary monocistronic inversion vector. The gene of interest (*goi*) coding region is flanked by inverted lox sites (triangles). CRE activates *goi* expression by inversion, so that the coding strand is transcribed. *rrn16*, *trnV* and *rps12/7* are plastid genes (Shinozaki et al. 1986).

Figure 13 shows an alternative dicistronic lox inversion vector. Note that the inverted lox sites flank the selective marker (*aadA*) and *goi*, and only one gene is expressed. *rrn16*, *trnV* and *rps12/7* are plastid genes (Shinozaki et al. 1986).

Figure 14 shows a basic tobacco plastid lox deletion vector. The vector provides is a suitable backbone for vector construction and targets insertions into the *trnV-rps12/7* intergenic region.

Figure 15 shows a tobacco plastid *lox* >*aadA*> deletion vector. *rrn16*, *trnV* and *rps12/7* are plastid genes (Shinozaki et al. 1986).

5

Figure 16 shows a tobacco constitutive >*aadA*>*goi* dicistronic deletion vector. *rrn16*, *trnV* and *rps12/7* are plastid genes and are described in (Shinozaki et al. 1986).

10

Figure 17 shows a tobacco constitutive *goi* >*aadA*> dicistronic deletion vector. Note that vectors shown in Fig. 16 and Fig. 17 differ in the relative order of marker gene and the gene of interest. *rrn16*, *trnV* and *rps12/7* are plastid genes (Shinozaki et al. 1986).

15

Figure 18 shows a tobacco constitutive *goi* >*aadA*> dicistronic deletion vector, in which expression of *aadA* is dependent on translational coupling. Note that in this construct only one leader sequence is utilized. *rrn16*, *trnV* and *rps12/7* are plastid genes (Shinozaki et al. 1986).

20

Figure 19 shows a tobacco inducible *lox* deletion vector. Expression of *goi* is dependent on *aadA* excision. *rrn16*, *trnV* and *rps12/7* are plastid genes (Shinozaki et al. 1986). Abbreviations: P, promoter; T, 3' untranslated region; L1 is 5' leader sequence.

25

Figure 20 shows a vector suitable for Cre-mediated deletion of *clpP* gene from the plastid genome. The region of engineered plastid genome shown is the sequence contained in the plastid transformation vector. The *clpP* Exons are dark boxes, the Introns are open

30

boxes. Map position of plastid genes *psbB*, *rps12* Exon I and *rpl20* is also shown.

DETAILED DESCRIPTION OF THE INVENTION

5 The following definitions are provided to aid in understanding the subject matter regarded as the invention.

 Heteroplastomic refers to the presence of a mixed population of different plastid genomes within a single plastid or in a population of plastids contained in
10 plant cells or tissues.

 Homoplastomic refers to a pure population of plastid genomes, either within a plastid or within a population contained in plant cells and tissues.

15 Homoplastomic plastids, cells or tissues are genetically stable because they contain only one type of plastid genome. Hence, they remain homoplastomic even after the selection pressure has been removed, and selfed progeny are also homoplastomic. For purposes of the present
20 invention, heteroplastomic populations of genomes that are functionally homoplastomic (i.e., contain only minor populations of wild-type DNA or transformed genomes with sequence variations) may be referred to herein as
 "functionally homoplastomic" or "substantially
25 homoplastomic." These types of cells or tissues can be readily purified to a homoplastomic state by continued selection.

 Plastome refers to the genome of a plastid.

30 Transplastome refers to a transformed plastid genome.

 Transformation of plastids refers to the stable integration of transforming DNA into the plastid genome that is transmitted to the seed progeny of plants containing the transformed plastids.

35 Selectable marker gene refers to a gene that upon

expression confers a phenotype by which successfully transformed plastids or cells or tissues carrying the transformed plastid can be identified.

5 Transforming DNA refers to homologous DNA, or heterologous DNA flanked by homologous DNA, which when introduced into plastids becomes part of the plastid genome by homologous recombination.

10 Operably linked refers to two different regions or two separate genes spliced together in a construct such that both regions will function to promote gene expression and/or protein translation.

15 "Nucleic acid" or a "nucleic acid molecule" as used herein refers to any DNA or RNA molecule, either single or double stranded and, if single stranded, the molecule of its complementary sequence in either linear or circular form. In discussing nucleic acid molecules, a sequence or structure of a particular nucleic acid molecule may be described herein according to the normal convention of providing the sequence in the 5' to 3' direction. With reference to nucleic acids of the invention, the term "isolated nucleic acid" is sometimes used. This term, when applied to DNA, refers to a DNA molecule that is separated from sequences with which it is immediately contiguous in the naturally occurring genome of the organism in which it originated. For example, an "isolated nucleic acid" may comprise a DNA molecule inserted into a vector, such as a plasmid or virus vector, or integrated into the genomic DNA of a prokaryotic or eukaryotic cell or host organism.

30 When applied to RNA, the term "isolated nucleic acid" refers primarily to an RNA molecule encoded by an isolated DNA molecule as defined above. Alternatively, the term may refer to an RNA molecule that has been sufficiently separated from other nucleic acids with which it would be associated in its natural state (i.e.,
35

in cells or tissues). An isolated nucleic acid (either DNA or RNA) may further represent a molecule produced directly by biological or synthetic means and separated from other components present during its production.

5 The terms "percent similarity", "percent identity" and "percent homology" when referring to a particular sequence are used as set forth in the University of Wisconsin GCG software program.

10 The term "functional" as used herein implies that the nucleic or amino acid sequence is functional for the recited assay or purpose.

 The phrase "consisting essentially of" when referring to a particular nucleotide or amino acid means a sequence having the properties of a given SEQ ID No.:. For example, when used in reference to an amino acid sequence, the phrase includes the sequence per se and molecular modifications that would not affect the basic and novel characteristics of the sequence.

20 A "replicon" is any genetic element, for example, a plasmid, cosmid, bacmid, phage or virus, that is capable of replication largely under its own control. A replicon may be either RNA or DNA and may be single or double stranded.

25 A "vector" is a replicon, such as a plasmid, cosmid, bacmid, phage or virus, to which another genetic sequence or element (either DNA or RNA) may be attached so as to bring about the replication of the attached sequence or element.

30 An "expression operon" refers to a nucleic acid segment that may possess transcriptional and translational control sequences, such as promoters, enhancers, translational start signals (e.g., ATG or AUG codons), polyadenylation signals, terminators, and the like, and which facilitate the expression of a
35 polypeptide coding sequence in a host cell or organism.

with the sequence of the target nucleic acid to anneal therewith specifically.

The term "primer" as used herein refers to an oligonucleotide, either RNA or DNA, either
5 single-stranded or double-stranded, either derived from a biological system, generated by restriction enzyme digestion, or produced synthetically which, when placed in the proper environment, is able to functionally act as an initiator of template-dependent nucleic acid
10 synthesis. When presented with an appropriate nucleic acid template, suitable nucleoside triphosphate precursors of nucleic acids, a polymerase enzyme, suitable cofactors and conditions such as a suitable temperature and pH, the primer may be extended at its 3'
15 terminus by the addition of nucleotides by the action of a polymerase or similar activity to yield an primer extension product. The primer may vary in length depending on the particular conditions and requirement of the application. For example, in diagnostic
20 applications, the oligonucleotide primer is typically 15-25 or more nucleotides in length. The primer must be of sufficient complementarity to the desired template to prime the synthesis of the desired extension product, that is, to be able anneal with the desired template
25 strand in a manner sufficient to provide the 3' hydroxyl moiety of the primer in appropriate juxtaposition for use in the initiation of synthesis by a polymerase or similar enzyme. It is not required that the primer sequence represent an exact complement of the desired
30 template. For example, a non-complementary nucleotide sequence may be attached to the 5' end of an otherwise complementary primer. Alternatively, non-complementary bases may be interspersed within the oligonucleotide primer sequence, provided that the primer sequence has
35 sufficient complementarity with the sequence of the

desired template strand to functionally provide a template-primer complex for the synthesis of the extension product. Amino acid residues described herein are preferred to be in the "L" isomeric form. However, residues in the "D" isomeric form may be substituted for any L-amino acid residue, provided the desired properties of the polypeptide are retained.

All amino-acid residue sequences represented herein conform to the conventional left-to-right amino-terminus to carboxy-terminus orientation.

The term "tag," "tag sequence" or "protein tag" refers to a chemical moiety, either a nucleotide, oligonucleotide, polynucleotide or an amino acid, peptide or protein or other chemical, that when added to another sequence, provides additional utility or confers useful properties, particularly in the detection or isolation, to that sequence. Thus, for example, a homopolymer nucleic acid sequence or a nucleic acid sequence complementary to a capture oligonucleotide may be added to a primer or probe sequence to facilitate the subsequent isolation of an extension product or hybridized product. In the case of protein tags, histidine residues (e.g., 4 to 8 consecutive histidine residues) may be added to either the amino- or carboxy-terminus of a protein to facilitate protein isolation by chelating metal chromatography.

Alternatively, amino acid sequences, peptides, proteins or fusion partners representing epitopes or binding determinants reactive with specific antibody molecules or other molecules (e.g., flag epitope, c-myc epitope, transmembrane epitope of the influenza A virus hemagglutinin protein, protein A, cellulose binding domain, calmodulin binding protein, maltose binding protein, chitin binding domain, glutathione S-transferase, and the like) may be added to proteins to

facilitate protein isolation by procedures such as affinity or immunoaffinity chromatography. Chemical tag moieties include such molecules as biotin, which may be added to either nucleic acids or proteins and facilitates isolation or detection by interaction with avidin reagents, and the like. Numerous other tag moieties are known to, and can be envisioned by, the trained artisan, and are contemplated to be within the scope of this definition.

As used herein, the terms "reporter," "reporter system", "reporter gene," or "reporter gene product" shall mean an operative genetic system in which a nucleic acid comprises a gene that encodes a product that when expressed produces a reporter signal that is a readily measurable, e.g., by biological assay, immunoassay, radioimmunoassay, or by colorimetric, fluorogenic, chemiluminescent or other methods. The nucleic acid may be either RNA or DNA, linear or circular, single or double stranded, antisense or sense polarity, and is operatively linked to the necessary control elements for the expression of the reporter gene product. The required control elements will vary according to the nature of the reporter system and whether the reporter gene is in the form of DNA or RNA, but may include, but not be limited to, such elements as promoters, enhancers, translational control sequences, poly A addition signals, transcriptional termination signals and the like.

The terms "transform", "transfect", "transduce", shall refer to any method or means by which a nucleic acid is introduced into a cell or host organism and may be used interchangeably to convey the same meaning. Such methods include, but are not limited to, transfection, electroporation, microinjection, PEG-fusion, biolistic bombardment and the like.

A "clone" or "clonal cell population" is a population of cells derived from a single cell or common ancestor by mitosis.

5 A "cell line" is a clone of a primary cell or cell population that is capable of stable growth *in vitro* for many generations.

CRE-MEDIATED SITE SPECIFIC RECOMBINATION

10 The plastid genome of higher plants is present in 100-10,000 copies per cell. Incorporation of a selectable marker gene is essential to ensure preferential maintenance of the transformed plastid genome copies carrying useful genes with no selectable phenotype. However, once transformation is accomplished, 15 maintenance of the marker gene is undesirable. In accordance with the present invention, a bacteriophage P1CRE-*loxP* site-specific recombination system is provided which is suitable for efficient elimination of marker genes from the plastid genome. The system 20 exemplified herein has two components: a plastid tester strain carrying a cytosine deaminase (*codA*) transgene flanked by *lox* sites conferring sensitivity to 5-fluorocytosine and a nuclear CRE line carrying a nuclear-encoded, plastid-targeted CRE. Both the plastid 25 tester (no CRE activity) and the nuclear CRE line (no *lox* sequence) were genetically stable. However, *codA* was eliminated at a very fast rate when the plastid-targeted CRE was introduced into the plastid tester strain by transformation or crossing. The gene for the nuclear- 30 encoded CRE was subsequently separated from the transformed plastids by segregation in the seed progeny. Excision of *codA* by CRE was often accompanied by deletion of a plastid genome segment flanked by short directly repeated sequences. Removal of the antibiotic 35 resistance marker from the transplastomic plants

eliminates the metabolic burden imposed by the expression of the selectable marker gene and should also improve public acceptance of the transgenic crops.

Additional applications of the CRE-lox site-specific recombination system are activation of plastid gene
5 expression by deletion or inversion of plastid genome sequences and induction of controlled cell death by deleting vital genes in the male reproductive tissue.

Although the use the CRE recombinase is exemplified
10 herein, other prokaryotic and eukaryotic site-specific recombinases would be equally suitable for the elimination of the marker genes.

Recently, several prokaryotic and lower eukaryotic site-specific recombination systems have been shown to
15 operate successfully in higher eukaryotes. In plant and animal cells functional site-specific recombination systems from bacteriophages P1 (Cre-lox) Mu (Gin-gix), and from the inversion plasmids of *Saccharomyces cerevisiae* (FLP-*frt*) (Morris et al. 1991; O'Gorman et
20 al. 1991; Lichtenstein and Barrena 1993; Lyznik et al. 1993; Lyznik et al. 1995; Lyznik et al. 1996) and *Zygosaccharomyces rouxii* (R-RS). In each of these systems, no additional factor aside from the recombinase and target sequences is required for recombination.

Reviewed in van Haaren and Ow, 1993. The CRE-loxP site-specific recombination system of bacteriophage P1 has
25 been studied extensively *in vitro* and in *E. coli* (Craig 1988; Adams et al. 1992). Expression of the CRE protein (38.5 kDa) is sufficient to cause recombination between
30 34 bp *loxP* sites that consist of 13 bp inverted repeats separated by 8 bp asymmetric spacer sequence. If there are two *loxP* sites within a DNA segment, the result of the recombination reaction depends on the relative position of the recombination sites. If the
35 recombination sites form a direct repeat, that if they

are in the same orientation, recombination results in deletion of the intervening DNA. If the recombination sites are in an inverted orientation, CRE-mediated recombination results in an inversion of the intervening DNA. The products of these reactions are shown in Fig. 1. The CRE site-specific recombination system has been employed for the elimination of nuclear genes in a number of eukaryotic systems, including higher plants (Dale and Ow 1991; Russell et al. 1992; Srivastava et al. 1999).

Before the present invention, the efficiency of CRE-mediated elimination of targeted plastid genes was unknown. To explore this system for this purpose, CRE-mediated elimination of the *codA* gene encoding cytosine deaminase (CD; EC 3.5.4.1) was assessed. Cytosine deaminase converts 5-fluorocytosine (5FC) into 5-fluorouracil (5FU), the precursor of 5-fluoro-dUMP. 5FC is lethal for CD-expressing cells due to irreversible inhibition of thymidylate synthase by 5-fluoro-dUMP (Beck et al. 1972). Cytosine deaminase is absent in plants. Expression of the bacterial *codA* in plastids renders cells sensitive to 5FC, while cells deficient in transgene expression are resistant (Serino and Maliga 1997). Thus, 5FC resistance could be used for positive identification of cells with CRE-induced *codA* deletion, even if such deletion events were relatively rare. The test system of the present invention incorporates a *codA* gene in the tobacco plastid genome between two directly oriented *lox* sites (>*codA*>). The transplastome was stable in the absence of CRE activity. However, highly efficient elimination of >*codA*> was triggered by introduction of a nuclear-encoded plastid-targeted CRE.

EXAMPLE 1

CRE-MEDIATED DELETION OF THE SELECTABLE PLASTID MARKER

Cre-mediated deletion of the selective plastid marker in the plastids of tobacco somatic cell is described in Example I. The selectable marker flanked by the lox sites is exemplified here by *codA*. However, it could be any other selectable and non-selectable marker gene, or any DNA sequence independent of information content flanked by lox sites in the plastid genome. Components of the test system are tobacco plants carrying a *codA* coding region flanked by lox sites (>*codA*>). A second component of the test system is a nuclear gene encoding a plastid targeted CRE-site specific recombinase. Deletion of a plastid encoded >*codA*> is achieved by introducing nuclear Cre into the nucleus of somatic (leaf) tobacco cells by *Agrobacterium*-mediated transformation. Alternatively, the nuclear encoded Cre gene may be introduced by fertilization with pollen of an appropriate activator-of-deletion strain. The nuclear Cre gene is subsequently removed by segregation in the seed progeny.

MATERIALS AND METHODS FOR THE PRACTICE OF EXAMPLE 1

The following materials and methods are provided to facilitate the practice of Example 1.

Plastid *codA* with direct lox sites.

The *codA* gene is contained in a *SacI-HindHIII* fragment. The gene map is shown in Fig. 2. *PrnloxD* (Seq. ID No. 4) is a plastid rRNA operon (*rrn16*) promoter derivative. It is contained in a *SacI-EcoRI* fragment obtained by PCR using oligonucleotides 5'-

GGGGAGCTCGCTCCCCGCCGTCGTTCAATG-3' and 5'-

GGAATTCATAACTTCGTATAGCATACATTATACGAAGTTAT

GCTCCCAGAAATATAGCCA-3' as primers and plasmid pZS176 (progenitor of plasmid pZS197; Svab and Maliga 1993) as a template. The promoter fragment *PrnrnloxD* contains a *lox* site at the 3' end adjacent to the *EcoRI* site. The *EcoRI-NcoI* fragment contains the ribosome binding site from plasmid pZS176. The fragment was obtained by annealing the complementary oligonucleotides 5'-AATTCGAAGCGCTTGGATACAGTTGTAGGGAGGGATC-3' and 5'-CATGGATCCCTCCCTACAACGTATCCAAGCGCTTCG-3'. The *codA* coding region is contained in an *NcoI-XbaI* fragment (Serino and Maliga 1997). The *TrbcLloxD* (Seq. ID No. 5) is the *rbcL* 3'-untranslated region contained in an *XbaI-HindIII* fragment obtained by PCR using oligonucleotides 5'-GGTCTAGATAACTTCGTATAATGTATGCTATA CGAAGTTATAGACATTAGCAGATAAATT-3' and 5'-GGGGGTACCAAGCTTGCTAGATTTTGTATTTCAAATCTTG-3' and plasmid pMSK48 (Khan and Maliga 1999) as template. *TrbcLloxD* contains a *lox* site adjacent to the *XbaI* site in direct orientation relative to the *lox* site in the *codA* 5'UTR. The chimeric *PrnrnloxD:codA:TrbcLloxD* gene was introduced into the tobacco plastid transformation vector pPRV111B (Zoubenko et al. 1994) as a *SacI-HindIII* fragment to obtain plasmid pSAC48.

Plastid-targeted nuclear *cre* linked to a nuclear kanamycin resistance gene. Two plastid targeted nuclear *cre* genes were tested. The *cre* gene in *Agrobacterium* binary vector pK027 and pK028 encode the CRE recombinase at its N terminus translationally fused with the pea Rubisco small subunit (SSU) chloroplast transit peptide (Timko et al. 1985) and twenty two and five amino acids of the mature Rubisco small subunit, respectively. Both *cre* genes are contained in an *EcoRI-HindIII* fragment. The schematic map of the genes is shown in Fig. 3. The

P2' *Agrobacterium* promoter (Velten et al. 1984) (Sequence ID. No.9) is contained in an *EcoRI*-*NcoI* fragment. The P2' promoter fragment was obtained by PCR using oligonucleotides 5'-
5 ccgaattcCATTTTCACGTGTGGAAGATATG-3' and 5'-
 ccccatggttaggcctatCGATTTGGTGTATCGAGATTGG-3' as primers and plasmid pHCl (Carrer et al. 1990) as template. PCR amplification introduced an *EcoRI* site at the 5' end and *ClaI*, *BamHI* and a *NcoI* sites at the 3' end. A T
10 introduced between the *ClaI* and the *BamHI* sites eliminates an ATG and introduces an in-frame stop codon (Sriraman 2000). The Rubisco SSU transit peptides are included in *BamHI*-*NcoI* fragments. The pKO27 fragment (Pea SSU-TP22; Sequence ID No. 7) was obtained by using
15 oligonucleotides 5'-CCGGATCCAATTCAACCACAAGAACTAAC-3' and 5'-GGGGCTAGCCATGGCAGGCCACACCTGCATGCAC-3' as primers and plasmid pSSUpGEM4 as the template (Timko et al. 1985). The pKO28 fragment (Pea SSU-TP5; Sequence ID No. 6) was obtained by using oligonucleotides 5'-
20 CCGGATCCAATTCAACCACAAGAACTAAC-3' and 5'-GGGGCTAGCCATGGTCAATGGGTTCAAATAGG-3' as primers and plasmid pSSUpGEM4 as the template (Timko et al. 1985). A pea SSU-TP with 23 amino acids of the mature polypeptide is shown in Sequence ID No. 8. The *cre* coding region
25 included in a *NcoI*-*XbaI* fragment (Sequence ID No. 3) was obtained by PCR amplification using the Cre1 5'-GGGGAGCTCCATGGCTAGCTCCAATTTACT
 GACCGTACAC-3' and Gre2 5'-GGGTCTAGACTAATCGCCATCCTCGAGCAGGCGCACCATTGC-3' oligonucleotides as primers and
30 DNA isolated from *Escherichia coli* strain BNN132 (ATCC number 47059) as template. The presence of *cre* gene in plant nuclear DNA was confirmed by PCR amplification with the Cre 1 and Cre3 oligonucleotides. The sequence of Cre3 oligonucleotide is 5'-TCAATCGATGAGTTGCTTC-3'.
35 The *Agrobacterium nos* terminator (Tnos) is included in a

*Xba*I-*Hind*III fragment (Svab et al. 1990). The plastid targeted nuclear *cre* genes were introduced as *Eco*RI-*Hind*III fragments into the pPZP212 *Agrobacterium* binary vectors (Hajdukiewicz et al. 1994) to obtain plasmids pK027 and pK028 with twenty two and five amino acids of the mature Rubisco SSU. A schematic map of the *Agrobacterium* vectors is shown in Fig. 3.

Transgenic plants. Plastid transformation using the biolistic protocol, selection of transplastomic tobacco clones (RMOP medium, 500 mg/L spectinomycin dihydrochloride) and characterization of the transplastomic clones by DNA gel blot analysis was described (Svab and Maliga 1993). Transformation with *Agrobacterium* vectors pK028 or pK027 and regeneration of transformed tobacco plants has also been reported (Hajdukiewicz et al. 1994). Briefly, nuclear gene transformants were selected by kanamycin resistance on RMOP shoot regeneration medium containing 100 mg/L kanamycin and 500 mg/L carbenicillin. Kanamycin resistance of the shoots was confirmed by rooting on plant maintenance (RM) medium containing 100 mg/L kanamycin. Testing of 5FC cytotoxicity was carried out on RMPO medium according to published procedures (Serino and Maliga 1997).

Transplastomic tobacco plants with a *codA* gene flanked by direct *lox* sites.

Plastid transformation vector pSAC48 carries a *codA* gene in which two *lox* sites flank the coding region in a direct orientation. If the *codA* coding region is deleted via the *lox* sites, a *lox* site flanked by the promoter (Prn) and terminator (TrbcL) are left behind. The selective marker in pSAC48, a pPRV111B vector

derivative, is a spectinomycin resistance (*aadA*) gene (Fig. 2). Transformation with plasmid pSCAC48 yielded a number of independently transformed transplastomic lines, of which four were purified to the homoplastomic state: Nt-pSAC48-21A, Nt-pSAC48-16C, Nt-pSAC48-16CS and Nt-pSAC48-9A. These lines are considered identical other than they have been generated independently. A uniform population of transformed plastid genomes in the transplastomic plants was verified by DNA gel blot analysis (see below).

Nuclear-encoded plastid-targeted Cre genes.

To activate deletion of the plastid *>codA>* gene we introduced an engineered *cre* gene into the nucleus of the transplastomic lines encoding a plastid-targeted CRE. Targeting of nuclear-encoded plastid proteins is by an N-terminal transit peptide (TP) cleaved off during import from the cytoplasm into plastids (Soll and Tien, 1998). To ensure plastid targeting of the CRE recombinase, it was translationally fused with the Rubisco small subunit (SSU) transit peptide (Timko et al. 1985). Therefore, the product of the protein fusion is SSU-TP-CRE. Efficiency of import of chimeric proteins depends on the size of mature protein N-terminus incorporated in the construct (Wasmann et al. 1986; Lubben et al. 1989). Two chimeric *cre* genes (*Ssu-tp-cre*) were prepared, one with 5 (vector pK028) and one with 22 (plasmid pK027) amino acids of the mature SSU N-terminus, encoding SSU-TP5-CRE and SSU-TP22-CRE, respectively. These genes are also referred to as *Cre1* and *Cre2*, respectively (Table 1). The *cre* genes were expressed in the P2' promoter and Tnos terminator cassettes in the *Agrobacterium* pPZP212 binary vector which carries kanamycin resistance (*neo*) as a selectable marker (Fig. 3).

Tobacco plant transformed with *Ssu-tp5-cre* (pK037) and *Ssu-tp22-cre* (pK036) were also obtained. In these plants the nuclear *cre* is expressed from the cauliflower mosaic virus 35S promoter (Seq. ID No. 10; Timmermans et al. 1990).

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Line	Plastid genotype ^a	Nuclear marker	
Wild-type	<i>trnV+</i> <i>aadA-</i> <i>codA-</i>		
Nt-pSAC48-21A	<i>trnV+</i> <i>aadA+</i> <i>codA+</i>		
Nt-pSAC48-16C	<i>trnV+</i> <i>aadA+</i> <i>codA+</i>		
Cre1-1	<i>trnV+</i> <i>aadA+</i> <i>codA-</i> <i>trnV-</i> <i>aadA-</i> <i>codA-</i>	<i>neo</i>	
Cre1-2	<i>trnV+</i> <i>aadA+</i> <i>codA-</i> <i>trnV-</i> <i>aadA-</i> <i>codA-</i>	<i>neo</i>	
Cre1-3	<i>trnV+</i> <i>aadA-</i> <i>codA-</i>	<i>neo</i>	
Cre1-4	<i>trnV-</i> <i>aadA-</i> <i>codA-</i>	<i>neo</i>	
Cre1-10	<i>trnV-</i> <i>aadA-</i> <i>codA-</i>	<i>neo</i>	
Cre2-1	<i>trnV+</i> <i>aadA+</i> <i>codA-</i>	<i>neo</i>	
Cre2-2	<i>trnV+</i> <i>aadA+</i> <i>codA-</i> <i>trnV+</i> <i>aadA*</i> <i>codA-</i> <i>trnV-</i> <i>aadA-</i> <i>codA-</i>	<i>neo</i>	
Cre2-3	<i>trnV+</i> <i>aadA+</i> <i>codA+</i> <i>trnV+</i> <i>aadA+</i> <i>codA-</i> <i>trnV+</i> <i>aadA*</i> <i>codA-</i> <i>trnV-</i> <i>aadA-</i> <i>codA-</i>	<i>neo</i>	
Cre2-4	<i>trnV+</i> <i>aadA+</i> <i>codA-</i>	<i>neo</i>	
Cre2-5	<i>trnV+</i> <i>aadA+</i> <i>codA-</i>	<i>neo</i>	
Cre2-10	<i>trnV+</i> <i>aadA+</i> <i>codA-</i> <i>trnV-</i> <i>aadA-</i> <i>codA-</i>	<i>neo</i>	
Cre1-100	<i>trnV+</i> <i>aadA-</i> <i>codA-</i>	<i>neo</i>	
Cre2-100	<i>trnV+</i> <i>aadA-</i> <i>codA-</i>	<i>neo</i>	
Cre2-200	<i>trnV+</i> <i>aadA-</i> <i>codA-</i>	<i>neo</i>	
Cre2-300	<i>trnV+</i> <i>aadA-</i> <i>codA-</i>	<i>neo</i>	

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^aPresence or absence of plastid gene is indicated by + or -. Since the plastid *trnV* gene is deleted in some of the lines, the wild-type plastid genotype is *trnV+* *aadA-* *codA-*.

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Deletion of *codA* from the plastid genome in somatic

cells.

To test the efficiency of CRE-mediated deletion in somatic cells, the *Ssu-tp-crc* genes were introduced into the nucleus of the transplastomic >codA> lines by cocultivation of *Agrobacterium* and tobacco leaf disks. Plants representing 11 individual *Ssu-tp-crc* insertion events have been characterized. Five lines (Cre1-derivatives) were obtained by transformation with *Ssu-tp5-crc* gene (vector pK028) and six lines (Cre2-derivatives) were obtained by transformation with the *Ssu-tp22-crc* (vector pK027) (Table 1).

Deletion of *codA* was first tested in a DNA sample taken from one leaf of eleven kanamycin resistant shoots representing an individual integration event of the nuclear Cre gene. Subsequently, 4 to 7 additional leaves were sampled from six shoots to confirm that the result of the analysis is typical for the plant.

The initial DNA samples were first screened for the loss of >codA> by PCR using the 01/02 primer pair

complementary to sequences in the *aadA* coding region N terminus and the *codA* promoter (Fig. 4A). Amplification with these primers yields a ~0.7-kb fragment if >codA> is deleted and a ~2.0-kb fragment if the >codA> gene is still present. Ethidium bromide stained gels of PCR

products in Fig. 5 indicate complete loss of >codA> in each of the samples. A perfect, reconstituted lox site between Prn and TrbcL was confirmed in eight clones by PCR amplification of the region with primers 01/04 from the same DNA samples and direct sequencing of the

amplification product with primer 02 (not shown). In two clones (Cre1-4, Cre1-10) a fragment is missing due to deletion of *aadA* alongside with *codA* (see below).

Plastid genome structure in the initial DNA sample was determined by gel blot analysis of *ApaI-EcoRV* digested

total cellular DNA. The probes were the plastid targeting region and the *aadA* and *codA* coding regions. The DNA gel blots are shown in Fig. 6. The maps of the parental genomes and deletion derivatives that help to interpret these genomes are shown in Fig. 4. In the plastid tester strains expressing no CRE (Nt-pSAC48-21A, Nt-pSAC48-16C) all three probes hybridized to the same 4.9-kb DNA fragment consistent with both *codA* and *aadA* being present in all the plastid genome copies. In the SSU-TP-CRE expressing plants no 4.9-kb fragment was detectable indicating the dramatic speed by which the *>codA>* gene was eliminated from the plastid genome. CRE-mediated deletion of *>codA>* via the *lox* sites yielded the 3.6-kb fragment detected in nine of the eleven clones. The 3.6-kb fragment was the only product detected in four clones, and was present in a heteroplastomic population in five clones. Unanticipated was formation of a 1.4-kb *ApaI-EcoRV* fragment in five clones. DNA gel blot analysis confirmed that this fragment lacks both *codA* and *aadA*, and is smaller than the wild type *ApaI-EcoRV* fragment (1.9-kb). Direct sequencing of PCR products in this region confirmed deletion of *codA*, *aadA* and *trnV* by homologous recombination via the duplicated *Prrn* promoter regions. One of the *Prrn* promoters is driving *codA*, the other is upstream of the rRNA operon at its native location. Deletion of *trnV* is the reason why the *ApaI-EcoRV* fragment derived from this region (1.4-kb) is smaller than the wild-type fragment (1.9-kb). The initial DNA samples were taken from one leaf of a plant obtained by rooting the shoot obtained after transformation with the *Ssu-tp-cre* genes. To confirm that the DNA samples extracted from the leaf were typical for the plant, we have sampled several more

leaves from the same plants (Fig. 7). In four clones *codA* was excised by CRE via the *lox* sites, and the shoots were homoplastomic for the deleted genome. Two of these, Cre1-3 and Cre2-4 were further characterized by testing seven and four additional leaves of the same plants, respectively. DNA gel blot analysis of these samples confirmed a uniform deletion of *>codA>* from all genome copies. These plants are the desired final products carrying the desired plastid transgenes and lacking the undesirable selective marker. These plants and their progeny can be used directly for the production of recombinant proteins as they are free from the selectable marker gene. Furthermore, these plants are a source of engineered chloroplasts for introduction into breeding lines by sexual crossing. The seed progeny of the plants is segregating for the *Ssu-tp-cre* activator gene. Plants with the desired chloroplasts but lacking the activator gene can be identified by PCR testing for *cre* sequences. Alternatively, individuals lacking *cre* can be identified in the seed progeny by sensitivity to kanamycin, since the *Ssu-tp-cre* genes in the pK027 and pK028 *Agrobacterium* vectors are physically linked to kanamycin resistance (*neo* gene; Fig. 3). In two clones, Cre1-4 and Cre1-10, deletion of *trnV* (encoding tRNA-Val^{GAC}), *aadA* and *codA* occurred by homologous recombination via the duplicated *Prn* promoter region. The Cre1-10 plant is homoplastomic for the deletion based on probing seven additional leaves (Fig. 7). Apparently, the one remaining *trnV* gene encoding tRNA-Val^{UAC} is sufficient for the translation of all valine codons, or there is import of tRNA-Val^{GAC} from the cytoplasm. In the Cre1-4 clone some of the leaves (two out of four) contained residual genome copies with *trnV* and *aadA*.

In five clones the initial DNA samples contained

more than one type of plastid genome copies. Mixed populations of plastid genome populations were confirmed in all parts of the plants by testing additional leaves (Fig. 7). Genetically stable *codA* deletion lines can be obtained from these heteroplastomic plants by testing plants regenerated from single somatic cells or individual seedlings in a segregating seed progeny.

Deletion of *codA* from the plastid genome in the seed progeny.

CRE-mediate deletion of the negative plastid marker *codA* in somatic cells was described in the previous section. Deletion of the plastid marker gene in the somatic cells of the transplastomic plants, without going through a sexual cycle, is highly desirable to accelerate the production of marker-free transplastomic plants.

However, this approach is feasible only if there is a system for tissue culture and plant regeneration from somatic cells. Such system is unavailable for the economically important cereal crops rice and maize. As an alternative to transformation of somatic cells, we developed CRE activator lines carrying a nuclear-encoded plastid-targeted Cre to be used as the source of Cre gene when used as a pollen parent. The tobacco CRE activator lines were obtained by transforming the nucleus of wild-type plants with SSU-TP-CRE constructs. Lines in which the Cre is linked to a nuclear kanamycin resistance gene in a wild-type cytoplasm are Cre1-100, Cre2-100, Cre2-200 and Cre2-300 (Table 1).

To activate deletion of *>codA>* in the seed progeny, tester plants Nt-pSAC48-21A and Nt-pSAC48-16C were emasculated to prevent self fertilization, and fertilized with pollen from the Cre2-200 and Cre2-300 activator lines. The activator lines are primary transgenic plants (T_0) segregating for the *Ssu-tp-cre*

of plastid genome copies is determined by DNA gel blot analysis (Carrer et al. 1993; Svab and Maliga 1993; Carrer and Maliga 1995) and can be visualized in UV light when expressing the green fluorescence protein in plastids (Khan and Maliga 1999). Homoplastomic, genetically stable plants are obtained during a second cycle of plant regeneration from the leaves of the regenerated plants or in the seed progeny. The cells of the *>codA>* tester strains carry a uniform population of plastid genome copies. Thus, the *Ssu-tp-cre* is introduced into the nuclear genome of a cell that is homoplastomic for *>codA>*. It was expected that the regenerated shoots would contain a mixed population of plastid genome copies. Instead, all plastid genome copies lack *>codA>*, an evidence for the enormous selection pressure by CRE activity against plastid genome copies that carry two *lox* sites. It is important that deletion of *>codA>* occurs in the absence of selection against *>codA>* by exposure to 5-fluorocytosine. Virtually complete elimination of *>codA>* may also be obtained when CRE activity is introduced by crossing, using pollen of an appropriate deletion activator strain. Deletion of the selectable marker in somatic cells is the preferred choice over elimination of the marker in the seed progeny. The most important advantage is time saving. Introduction of *Ssu-tp-cre* into the nucleus of somatic cells requires only three to six weeks; *Ssu-tp-cre* segregates out in the first seed progeny. In contrast, introduction and elimination of *Ssu-tp-cre* takes one additional seed progeny, about three months.

Interestingly, genome copies with one *lox* site or no *lox* site (wild-type) are stable in CRE-expressing cells. Instability of genomes with two *lox* sites may be due to formation of linear ends during the excision

form lacking the N-terminal extension is targeted to the nucleus (Small et al. 1998). It was fortunate, that the Rubisco SSU N-terminal transit peptide dominated the CRE nuclear localization signals and the TP-CRE fusion protein was directed to plastids (chloroplasts).

A second property that is important for the present invention is maintenance of recombinase activity when CRE is fused with proteins or peptides at its N and C termini. N-terminal fusion of CRE with the *E. coli* maltose binding protein did not interfere with recombinase function (Kolb and Siddell 1996). CRE was also shown to accept a C-terminal fusion with GFP (Le et al. 1999) as well as an 11-amino-acid epitope to the herpes simplex virus (HSV) glycoprotein D coat protein. The epitope tag facilitates detection of CRE expression *in vitro* and *in vivo* using immunofluorescent labeling with a commercially available antibody (Stricklett et al. 1998). Apparently, the five and 22 amino acids that are left behind after processing of the SSU-TP5-CRE and SU-TP22-CRE proteins did not interfere with CRE function.

Dominant negative selection markers for positive identification of deletion derivatives. A practical application of the present invention is the removal of selectable marker genes from the transformed plastid genome. In tobacco, the excision process mediated by the CRE constructs described herein is so efficient that the >codA< deletion derivatives can be identified in the absence of 5FC selection. However, in other crops CRE-mediated excision of marker genes may be less efficient. In these species, the positive selective marker (*aadA*) may be fused with a dominant negative selective marker using linker peptides as described in the literature (Khan and Maliga 1999) or the positive and negative

marker genes may be combined in a dicistronic operon (Staub and Maliga 1995). Dominant negative selection markers allow normally non-toxic compounds to be used as toxic agents, so that cells which express these markers are non-viable in the presence of the compound, while cells that don't carry them are unaffected.

For example, cytosine deaminase is absent in plants. Expression of *codA*, encoding cytosine deaminase (CD; EC 3.5.4.1), in plastids renders tissue culture cells and seedlings sensitive to 5FC, facilitating direct identification of clones lacking this negative selective marker (Serino and Maliga 1997). Cytosine deaminase converts 5-fluorocytosine (5FC) into 5-fluorouracil (5FU), the precursor of 5-fluoro-dUMP. 5FC is lethal for CD-expressing cells due to irreversible inhibition of thymidylate synthase by 5-fluoro-dUMP (Beck et al. 1972). We have found that seedlings and plant tissues expressing *>codA>* were sensitive to 5FC. Seedlings lacking *codA* could be readily identified by 5FC resistance. Thus, the constructs described here are suitable to express cytosine deaminase at sufficiently high levels to be useful to implement a negative selection scheme.

Alternative negative selective markers can be obtained by adaptation of substrate-dependent negative selection schemes described for nuclear genes. Such negative selection schemes are based on resistance to indole, naphthyl, or naphthalene acetamide (Depicker et al. 1988; Karlin-Neumann et al. 1991; Sundaresan et al. 1995), chlorate (Nussaume et al. 1991), kanamycin (Xiang and Guerra 1993) and 5-fluorocytosine (5FC) (Perera et al. 1993; Stougaard 1993).

EXAMPLE 2

Cre-MEDIATED INVERSION OF PLASTID DNA SEQUENCES

If the lox sites in bacteria are in an inverted orientation, CRE-mediated recombination results in an inversion of the intervening DNA. We have tested, whether the CRE-mediated inversion reaction also occurs in plastids of higher plants containing DNA sequences flanked by inverted lox sites. This was assessed using a kanamycin-resistance (>neo<) coding region in an inverted orientation relative to the promoter (Fig. 9)... In this construct the non-coding strand of neo is transcribed and the plants are kanamycin sensitive. The >neo< coding region is flanked by inverted lox sites. CRE-mediated inversion of the sequences reverses neo orientation resulting in the transcription of the sense strand and expression of kanamycin resistance. Inversion of the plastid-encoded >neo< coding region may be achieved by multiple approaches. One approach is to introduce a nuclear Cre into the nucleus of somatic tobacco cells, e.g., leaf, by *Agrobacterium*-mediated transformation. A second approach is introduction of the nuclear-encoded Cre gene by fertilization with pollen of an appropriate activator-of-inversion strain. Additional approaches are to provide CRE-activity via the incorporation of chemically inducible promoter into the construct, or to transiently express CRE from a nuclear or chloroplast construct.

MATERIALS AND METHODS FOR THE PRACTICE OF EXAMPLE 2

Plastid neo gene with inverted lox sites. The neo gene is contained in a *SacI-HindHIII* fragment. The gene map is shown in Fig. 8. PrnrloxI (Seq. ID No. 1) is a plastid rRNA operon (rrn16) promoter derivative. It is contained in a *SacI-XbaI* fragment obtained by PCR using oligonucleotides 5'-ggggagctcGCTCCCCGCGTCGTTCAATG-3' and 5'-gggtctagataaacttcgtatagcatatacgaagttatGCTCCC AGAAATATAGCCA-3' as primers and plasmid pZS176

(progenitor of plasmid pZS197; Svab and Maliga 1993) as a template. The promoter fragment *PrrnloxI* contains a *lox* site at the 3' end adjacent to the *XbaI* site. The *neo* coding region is contained in an *NcoI-XbaI* fragment derived from plasmid pHC62. The *neo* sequence in plasmid pHC62 is identical with the *neo* sequence shown in Fig. 28B, US Patent 5,877,402. The *EcoRI-NcoI* fragment contains the ribosome binding site from plasmid pZS176. The fragment was obtained by annealing the complementary oligonucleotides 5'-
AATTCGAAGCGCTTGGATACAGTTGTAGGGAGGGATC-3' and 5'-
CATGGATCCCTCCCTACAACGTATCCAAGCGCTTCG-3'. The *TrbcLloxI* (Seq. ID No. 2) is the *rbcL* 3'-untranslated region contained in an *EcoRI-HindIII* fragment obtained by PCR using oligonucleotides 5'-gggaattcataacttcgtatagcatatatacgaagttatAGACATTAGCAGATAAATT-3' and 5'-
gggggtaccaagcttgCTAGATTTTGTATTTCAAATCTTG-3' and plasmid pMSK48 (Khan and Maliga 1999) as template. *TrbcLloxI* contains a *lox* site adjacent to the *EcoRI* site in an inverted orientation relative to the *lox* site in *PrrnloxI*. The chimeric *PrrnloxI:neo:TrbcLloxI* gene was introduced into the tobacco plastid transformation vector pPRV111B (Zoubenko et al. 1994) as a *SacI-HindIII* fragment to obtain plasmid pSAC38.

Plastid-targeted nuclear *cre* linked to a nuclear gentamycin resistance (*aacC1*) gene. The plastid targeted nuclear *cre* genes were introduced as *EcoRI-HindIII* fragments into the pPZP222 *Agrobacterium* binary vectors which carry a plant-selectable gentamycin resistance gene (Hajdukiewicz et al. 1994) to obtain plasmids pK030 and pK031 with twenty two and five amino acids of the mature Rubisco SSU. The map of the *Agrobacterium* vectors is identical with the one shown in Fig. 3. other than

they carry a gentamycin resistance gene.

Transplastomic tobacco plants with a neo gene flanked by inverted lox sites.

Plastid transformation vector pSAC38 with the inverted >neo< gene is shown in Fig. 9. The inverted >neo< gene was introduced into plastids by selection for spectinomycin resistance (aadA) encoded in the vector. Two independently transformed lines were purified to the homoplastomic state: Nt-pSAC38-9A and Nt-pSAC38-10C. The homoplastomic state was confirmed by DNA gel blot analysis.

Nuclear-encoded plastid-targeted Cre genes.

Plant activator lines in which *Ssu-tp-cre* is linked to a nuclear kanamycin resistance gene have been described in Example 1. The plastid marker to test CRE-activated inversion described in Example 2 utilizes a kanamycin resistance gene. Kanamycin resistance conferred by the plastid gene due to CRE-mediated inversion could not be distinguished from kanamycin resistance conferred by the marker gene of the *Agrobacterium* binary vector that was used to introduce the nuclear *cre*. Therefore, we have constructed activator strains in which *Ssu-tp-cre* is linked to gentamycin resistance. The *Ssu-tp22-cre* gene linked to the nuclear gentamycin resistance is the Cre3 strain and the *Ssu-tp5-cre* gene linked to gentamycin resistance is the Cre4 strain.

Inversion of >neo< in the plastid genome of somatic cells.

The nuclear *cre* genes were introduced into the

chloroplast >neo< tester strains by cocultivation of tobacco leaves with the *Agrobacterium* strains and selection for gentamycin resistance (100 mg/L). Digestion of total cellular DNA with BamHI and probing with the plastid targeting region (ApaI-EcoRV fragment, Fig. 4) hybridizes to 1.8-kb and a 3.8-kb fragments in the parental Nt-pSAC38-10C lines (Fig. 10). Activation by CRE in lines Cre3-3 and Cre4-5 created a mixed population of >neo< genes representing the original and inverted orientations detected as the original 3.8-kb and 1.8-kb and the newly created 4.6-kb and 0.9-kb hybridizing fragments. Lines carrying the cre and an approximately wild-type size fragment are *aadA-neo* deletion derivatives, similar to those shown in Fig. 4. Thus, it appears that CRE mediated inversion via *lox* sites creates increased local recombination frequencies that leads to deletion of the transgenes via the short direct repeats of *Prn* promoters.

Controlling inversion via *lox* sites by CRE activity.

Here we describe constructs for CRE-mediated inversion of plastid genome segments flanked by inverted *lox* sites. Inversion of the sequences is independent of the encoded genetic information and relies only on CRE activity. CRE activity may be provided transiently, by expression in plastids from plastid signals described in US patent 5,877,402, or from nuclear genes encoding a plastid-targeted CRE. Such plastid-targeted CRE constructs are described in Example 1, for example the *Ssu-tp5-cre* or *Sssu-tp22-cre* genes. Alternative approaches to provide CRE activity are stable incorporation of a plastid-targeted nuclear Cre into the nucleus of somatic (leaf) cells by *Agrobacterium*-mediated, PEG induced or biolistic

transformation or by fertilization with pollen from a transformed plant. The Agrobacterium P2 promoter and cauliflower mosaic virus 35S promoter exemplified here are constitutive promoters. Regulated expression of CRE
5 may be important for certain applications.

Developmentally timed expression may be obtained from promoters with tissue specific activity. Regulated expression of CRE may be obtained from chemically induced nuclear gene promoters responding to elicitors,
10 steroids, copper or tetracycline (reviewed in; Gatz et al. 1992; Mett et al. 1993; Aoyama and Chau 1997; Gatz 1997; Martinez et al. 1999; Love et al. 2000) and described in US patent 5,614,395.

15 **Controlled expression of deleterious gene products**

There are a variety of valuable heterologous proteins that interfere with plastid metabolism. For example, certain proteins may be inserted into photosynthetic membranes and interfere with
20 photosynthesis. This problem can be circumvented by first growing the plants to maturity, then activating production of the deleterious protein by chemically inducing CRE expression. CRE, in turn, will make the gene expressible by *lox*-mediated inversion of the coding
25 region.

The molecular tools necessary for the construction of such plastid genes are described in present application. In case of the monocistronic inversion vector the gene of interest (*goi*) is flanked by inverted
30 *lox* sites and is introduced by linkage with *aadA* (Fig. 12). The selectable marker (*aadA*) coding region is the first reading frame, and is expressed from the promoter. The *goi* reading frame is the second coding region, and it is not expressed as it is in an inverted orientation
35 relative to the promoter. Expression of *goi* is induced

by CRE-mediated inversion of the *goi* coding region, as described for >neo< in Example 2 and is shown in Fig. 12.

The dicistronic lox inversion vector is shown in Fig. 13. In this case the inverted lox sites flank both *aadA* and *goi*. The selectable marker (*aadA*) coding region is expressed from the promoter. The *goi* reading frame is not expressed as it is in an inverted orientation relative to the promoter. Expression of *goi* is induced by CRE-mediated inversion of the *aadA*-*goi* containing region that results in simultaneous expression of *goi* and inactivation of *aadA*.

The presence of two lox sites may destabilize the plastid genome that leads to CRE-independent deletion of plastid genome sequences. However, it appears that CRE activity by itself is not mutagenic, and the plastid genomes are stable if only one lox site is present. Mutant lox sites that are efficiently excised but recombine into excision resistant sites have been described (Hoess et al. 1982; Albert et al. 1995). Such lox sites would mediate efficient inversion, but the new lox sites would be resistant to additional cycles of CRE activation. Providing only a short burst of CRE activation using a chemically induced promoter could further refine the expression system.

EXAMPLE 3

CRE-MEDIATED DELETION TO OBTAIN MARKER FREE TRANSPLASTOMIC PLANTS AND FOR HIGH LEVEL EXPRESSION OF THE RECOMBINANT PROTEINS

Plastid loxP vectors in this section are described for CRE-mediated excision of selective markers in transplastomic plants. Since excision of sequences

between directly oriented lox sites is very efficient, variants of the same vectors can be used for CRE-activated expression of recombinant proteins. A family of plastid vectors with suitably positioned lox sites is shown schematically in Fig. 14 through Fig. 17.

The map of the basic tobacco plastid lox deletion vector is shown in Fig. 14. It contains (a) two directly oriented lox sites separated by a unique BglIII cloning site and (b) an adjacent polycloning site. These sequences (Seg. ID No. 11) are inserted into the ScaI site plastid repeat vector pPRV100 (US Patent 5,877,402; Zoubenko et al. 1994). Suitable marker genes (*aadA*, *neo* or *kan*, *bar*, glyphosate resistance, bromoxynil resistance) for insertion into the BglIII site have been described in US Patent 5,877,402, WO 00/07421 and WO 00/03022.

The map of the tobacco plastid lox >aadA> deletion vector is shown in Fig. 15. It is the basic lox deletion vector with an *aadA* gene cloned into the BglIII sites oriented towards the *rrn* operon.

Maps of constitutive lox dicistronic deletion vectors are shown in Fig. 16 through Fig. 18. This dicistronic design enables simultaneous expression of both the first and the second open reading frames. The selectable marker designed for excision may be encoded in the first (Fig. 16) or second (Fig. 17, Fig. 18) open reading frames. Since a minimally 34 bp lox site is located between the two reading frames, both the marker gene (*aadA*) and the gene of interest have their own leader sequence to facilitate translation (Fig. 16, Fig. 17). Translational coupling may also be feasible if the lox site is incorporated in the marker gene coding region N terminus (Fig. 18). DNA sequence of promoter-lox constructs shown in Figs. 16 is set forth in Seq. ID

No. 1. Promoters and promoter-leader combinations suitable to promote high-level protein expression in plastids are described in European Patent Applications WO 00/07421, WO 97/06250 and WO 98/55595. Sequences suitable for directly oriented *lox* sites are given in Seq. ID No. 11. Translational coupling between a gene of interest and the downstream *aadA* is shown in Fig. 18. There are multiple ways of achieving translational coupling between adjacent genes (Baneyx 1999). One approach is incorporation of a properly spaced ribosome binding-site in the upstream gene's coding region (Schoner et al. 1986; Omer et al. 1995). An example for a suitable sequence directly upstream of the translation initiation codon (ATG) would be G-GAG-GAA-TAA-CTT-ATG. A specific example for the use of the sequence is translational coupling between a *bar* (suitable source described in European Patent Application WO 00/07421) and a downstream *aadA* are given in Seq. ID No. 12. Note *SalI* site downstream of AUG incorporated to facilitate engineering the *BglII*-*SalI* region and the directly oriented *lox* sites in the *aadA* coding region and downstream of *aadA*. The sequence is given for a *BglII*-*SpeI* fragment. The *BglII* site is within the *bar* coding region; the *SpeI* site is downstream of the second *lox* site, as marked in Fig. 18. If a C-terminal extension to create a ribosome binding site is unacceptable, a suitable sequence may be obtained by silent mutagenesis of the coding region at the third codon position. Variants of plastid ribosome binding sites have been catalogued (Bonham-Smith and Bourque 1989)

A tobacco inducible *lox* deletion vector is shown in Fig. 19. The marker gene (*aadA*) is encoded in the first reading frame, followed by a silent *goi* lacking the translation initiation codon (ATG) and the 5'

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1998) or the *trnV* gene the deletion of which has been described in Example 1. Excision of these genes is unlikely to cause cell ablation. The photosynthetic genes are essential for survival under field conditions. However, pigment deficient, non-photosynthetic plants can be maintained as long as they are grown on a sucrose-containing medium, or are grafted onto photosynthetically active wild-type (green) plants (Kanevski and Maliga 1994). Some of the house-keeping genes, such as the genes encoding the plastid multisubunit RNA polymerase are essential for photosynthetic growth, but not for survival (Allison et al. 1996). Thus, deletion of these genes is not suitable to trigger cell death. Only a relatively small number of plastid genes have proven to be essential for viability. The essential nature of the genes was recognized by the lack of homoplastomic cells in gene disruption experiments indicating that the loss of these genes results in cellular death. Cellular death due to lack of plastid function is understandable, as plastids are the site of the biosynthesis of amino acids, several lipids and are required for nitrate assimilation. Examples of plastid genes essential for cellular survival are the *clpP* protease subunit gene (Huang et al. 1994), *ycf1* and *ycf2*, the two largest plastid-encoded open reading frames (Drescher et al. 2000).

To induce cellular death by CRE-mediated excision, directly oriented *lox* sites can be incorporated in the plastid genome flanking essential genes, as shown for *clpP* in Fig. 20. The *clpP* gene has two large introns (807 bp and 637 bp) and the region can be conveniently cloned as a *SalI*-*SphI* fragment. The selectable marker *aadA* is inserted into a *KpnI* restriction site created by PCR mutagenesis downstream of *clpP* Exon 3, oriented towards *rps12* Exon I. One of the *lox* sites is

ACGTGCAAAA CAGGCTCTAG CGTTCGAACG CACTGATTTC GACCAGGTTC
 GTTCACTCAT GGAAAATAGC GATCGCTGCC AGGATATACG TAATCTGGCA
 TTTCTGGGGA TTGCTTATAA CACCCTGTTA CGTATAGCCG AAATTGCCAG
 GATCAGGGTT AAAGATATCT CACGTACTGA CCGTGCGGAGA ATGTTAATCC
 5 ATATTGGCAG AACGAAAACG CTGGTTAGCA CCGCAGGTGT AGAGAAGGCA
 CTTAGCCTGG GGGTAACTAA ACTGGTCGAG CGATGGATTT CCGTCTCTGG
 TGTAGCTGAT GATCCGAATA ACTACCTGTT TTGCCGGGTC AGAAAAAATG
 GTGTTGCCGC GCCATCTGCC ACCAGCCAGC TATCAACTCG CGCCCTGGAA
 GGGATTTTGT AAGCAACTCA TCGATTGATT TACGGCGCTA AGGATGACTC
 10 TGGTCAGAGA TACCTGGCCT GGTCTGGACA CAGTGCCCGT GTCGGAGCCG
 CGCGAGATAT GGCCCGCGCT GGAGTTTCAA TACCGGAGAT CATGCAAGCT
 GGTGGCTGGA CCAATGTAAA TATTGTCATG AACTATATCC GTAACCTGGA
 TAGTGAAACA GGGGCAATGG TGCGCCTGCT cGAgGATGGC GATTAGtctaga

SEQ. No. 4: PrnrloxD. Sequence

gagctcGCTCCCCCGCCGTCGTTCAATGAGAATGGATAAGAGGCTCGTGGGATTGA
 CGTGAGGGGGCAGGGATGGCTATATTTCTGGGAGCataacttcgtataatgtatgc
 20 tatacgaagttatgaattc

SEQ. No. 5: TrbcLloxD. sequence

tctagataacttcgtataatgtatgctatacgaagttatAGACATTAGCAGATAAAA
 25 TTAGCAGGAAATAAAGAAGGATAAGGAGAAAGAACTCAAGTAATTATCCTTCGTTTC
 TCTTAATTGAATTGCAATTAAACTCGGCCCAATCTTTTACTAAAAGGATTGAGCCG
 AATACAACAAAGATTCTATTGCATATATTTTGACTAAGTATATACTTACCTAGATA
 TACAAGATTTGAAATACAAAATCTAGcaagcttggtacc

SEQ. No. 6: Pea ssuTP5. sequence

ccggatccAA TTCAACCACA AGAACTAACA AAGTCAGAAA AATGGCTTCT
 ATGATATCCT CTTCCGCTGT GACAACAGTC AGCCGTGCTT CTAGGGTGCA
 35 ATCCGCGGCA GTGGCTCCAT TCGGCGGCCT GAAATCCATG ACTGGATTCC
 CAGTGAAGAA GGTCAACACT GACATTACTT CCATTACAAG CAATGGTGGA
 AGAGTAAAGT GCATGCAGGT GTGGCCTgcc atggctagc

SEQ. No. 7: Pea ssuTP22. sequence

ccggatcc AA TTCAACCACA AGAACTAACA AAGTCAGAAA AATGGCTTCT
 ATGATATCCT CTTCCGCTGT GACAACAGTC AGCCGTGCTT CTAGGGTGCA
 ATCCGCGGCA GTGGCTCCAT TCGGCGGCCT GAAATCCATG ACTGGATTCC
 45 CAGTGAAGAA GGTCAACACT GACATTACTT CCATTACAAG CAATGGTGGA
 AGAGTAAAGT GCATGCAGGT GTGGCCTCCA ATTGGAAAGA AGAAGTTTGA
 GACTCTTTCC TATTGCCCAC CATTGACCat ggctagc

SEQ. No. 8: Pea ssuTP23. sequence

ccggatccAA TTCAACCACA AGAACTAACA AAGTCAGAAA AATGGCTTCT
 ATGATATCCT CTTCGCTGT GACAACAGTC AGCCGTGCTT CTAGGGTGCA
 ATCCGCGGCA GTGGCTCCAT TCGGCGGCCT GAAATCCATG ACTGGATTCC
 CAGTGAAGAA GGTC AACACT GACATTACTT CCATTACAAG CAATGGTGGA
 5 AGAGTAAAGT GCATGCAGGT GTGGCCTCCA ATTGGAAAGA AGAAGTTTGA
 GACTCTTTC TATTTGCCAC CATTGACCAG AGATCAGTTG gctagcgg

SEQ. No. 9: P2 promoter sequence

10 gaattCATTT TCACGTGTGG AAGATATGAA TTTTTTTGAG AAAC TAGATA
 AGATTAATGA ATATCGGTGT TTTGGTTTTT TCTTGTTGGCC GTCTTTGTTT
 ATATTGAGAT TTTTCAAATC AGTGC GCAAG ACGTGACGTA AGTATCTGAG
 CTAGTTTTTA TTTTCTACT AATT TGGTCG TTTATTTTCGG CGTGTAGGAC
 ATGGCAACCG GGCCTGAATT TCGCGGGTAT TCTGTTTCTA TTCCAAC TTT
 15 TTCTTGATCC GCAGCCATTA ACGACTTTTG AATAGATACG CTGACACGCC
 AAGCCTCGCT AGTCAAAAGT GTACCAAACA ACGCTTTACA GCAAGAACGG
 AATGCGCGTG ACGCTCGCGG TGACGCCATT TCGCCTTTTC AGAAATGGAT
 AAATAGCCTT GCTTCCTATT ATATCTTCCC AAATTACCAA TACATTACAC
 TAGCATCTGA ATTTCATAAC CAATCTCGAT ACACCAAATC GATaggatcc
 20 taccatgg

SEQ. No. 10: 35S promoter sequence

AAGCTTGCCA ACATGGTGGA GCACGACACT CTCGTCTACT CCAAGAATAT
 25 CAAAGATACA GTCTCAGAAG ACCAAAGGGC TATTGAGACT TTTCAACAAA
 GGGTAATATC GGGAAACCTC CTCGGATTCC ATTGCCCAGC TATCTGTCAC
 TTCATCAAAA GGACAGTAGA AAAGGAAGGT GGCACCTACA AATGCCATCA
 TTGCGATAAA GGAAAGGCTA TCGTTCAAGA TGCCCTCTGCC GACAGTGGTC
 CCAAAGATGG ACCCCCACCC ACGAGGAGCA TCGTGGA AAAA AGAAGACGTT
 30 CCAACCACGT CTTCAAAGCA AGTGGATTGA TGTGATAACA TGGTGGAGCA
 CGACACTCTC GTCTACTCCA AGAATATCAA AGATACAGTC TCAGAAGACC
 AAAGGGCTAT TGAGACTTTT CAACAAAGGG TAATATCGGG AAACCTCCTC
 GGATTCCATT GCCCAGCTAT CTGTCACTTC ATCAAAAGGA CAGTAGAAAA
 GGAAGGTGGC ACCTACAAAT GCCATCAT TG CGATAAAGGA AAGGCTATCG
 35 TTCAAGATGC CTCTGCCGAC AGTGGTCCCA AAGATGGACC CCCACCCACG
 AGGAGCATCG TGGAAAAAGA AGACGTTCCA ACCACGTCTT CAAAGCAAGT
 GGATTGATGT GATATCTCCA CTGACGTAAG GGATGACGCA CAATCCC ACT
 ATCCTTCGCA AGACCCTTCC TCTATATAAG GAAGTTCATT TCATTTGGAG
 AGGACACGCT GAAATCACCA GTCTCTCTCT ACAAATCTAT CTCTCTCGAT
 40 TCGCGAGCTC GGTACCCGGG gatcgatcc

SEQ. No. 11: KpnI-lox-BglIII-lox-HindIII fragment

45 ggtaccATAACTTCGTATAATGTATGCTATACGAAGTTATagatctATAACTTCGT
 ATAATGTATGCTATACGAAGTTATaagctt

Seq. ID No. 12. Translational coupling of *bar* and *aadA*
 according to scheme in Fig. 18. BglIII-SpeI fragment.

50 GAGATCTGgg aggaataact tATGgggggtc gacATAACTT CGTATAATGT
 ATGCTATACG AAGTTATtaG AAGCGGTGAT CGCCGAAGTA TCGACTCAAC

5	TATCAGAGGT	AGTTGGCGTC	ATCGAGCGCC	ATCTCGAACC	GACGTTGCTG
	GCCGTACATT	TGTACGGCTC	CGCAGTGGAT	GGCGGCCTGA	AGCCACACAG
	TGATATTGAT	TTGCTGGTTA	CGGTGACCGT	AAGGCTTGAT	GAAACAACGC
	GGCGAGCTTT	GATCAACGAC	CTTTTGGA	CTTCGGCTTC	CCCTGGAGAG
	AGCGAGATTC	TCCGCGCTGT	AGAAGTCACC	ATTGTTGTGC	ACGACGACAT
	CATTCCGTGG	CGTTATCCAG	CTAAGCGCGA	ACTGCAATTT	GGAGAATGGC
	AGCGCAATGA	CATTCTTGCA	GGTATCTTCG	AGCCAGCCAC	GATCGACATT
	GATCTGGCTA	TCTTGCTGAC	AAAAGCAAGA	GAACATAGCG	TTGCCTTG
10	AGGTCCAGCG	GCGGAGGAAC	TCTTTGATCC	GGTTCCTGAA	CAGGATCTAT
	TTGAGGCGCT	AAATGAAACC	TTAACGCTAT	GGAACTCGCC	GCCCGACTGG
	GCTGGCGATG	AGCGAAATGT	AGTGCTTACG	TTGTCCCGCA	TTTGGTACAG
	CGCAGTAACC	GGCAAATCG	CGCCGAAGGA	TGTCGCTGCC	GACTGGGCAA
15	TGGAGCGCCT	GCCGGCCCAG	TATCAGCCCG	TCATACTTGA	AGCTAGACAG
	GCTTATCTTG	GACAAGAAGA	AGATCGCTTG	GCCTCGCGCG	CAGATCAGTT
	GGAAGAATTT	GTCCACTACG	TGAAAGGCGA	GATCACCAAG	GTAGTCGGCA
	AATAAATAAC	TTCGTATAAT	GTATGCTATA	CGAAGTTATa	ctagt

Seq. ID No. 13. CRE-induced expression of recombinant protein according to design in Fig. 19. SacI-NheI fragment.

	gagctcGCTC	CCCCGCCGTC	GTTCAATGAG	AATGGATAAG	AGGCTCGTGG
	GATTGACGTG	AGGGGGCAGG	GATGGCTATA	TTTCTGGGAG	AATTAACCGA
	TCGACGTGCa	AGCGGACATT	TATTTTtAAT	TCGATAATTT	TTGCAAAAAC
25	ATTTTCGACAT	ATTTATTTTAT	TTTATTATTA	TGgggATAAC	TTCGTATAAT
	GTATGCTATA	CGAAGTTATt	aGAAGCGGTG	ATCGCCGAAG	TATCGACTCA
	ACTATCAGAG	GTAGTTGGCG	TCATCGAGCG	CCATCTCGAA	CCGACGTTGC
	TGGCCGTACA	TTTGTACGGC	TCCGCAGTGG	ATGGCGGCCT	GAAGCCACAC
	AGTGATATTG	ATTTGCTGGT	TACGGTGACC	GTAAGGCTTG	ATGAAACAAC
30	GCGGCGAGCT	TTGATCAACG	ACCTTTTGGG	AACTTCGGCT	TCCCCCTGGAG
	AGAGCGAGAT	TCTCCGCGCT	GTAAGAAGTCA	CCATTGTTGT	GCACGACGAC
	ATCATTCCGT	GGCGTTATCC	AGCTAAGCGC	GAACTGCAAT	TTGGAGAATG
	GCAGCGCAAT	GACATTCTTG	CAGGTATCTT	CGAGCCAGCC	ACGATCGACA
	TTGATCTGGC	TATCTTGCTG	ACAAAAGCAA	GAGAACATAG	CGTTGCCTTG
35	GTAGGTCCAG	CGGCGGAGGA	ACTCTTTGAT	CCGGTTCTCT	AACAGGATCT
	ATTTGAGGCG	CTAAATGAAA	CCTTAACGCT	ATGGAACCTG	CCGCCGACT
	GGGCTGGCGA	TGAGCGAAAT	GTAGTGCTTA	CGTTGTCCCG	CATTTGGTAC
	AGCGCAGTAA	CCGCGAAAAT	CGCGCCGAAG	GATGTCGCTG	CCGACTGGGC
	AATGGAGCGC	CTGCCGGCCC	AGTATCAGCC	CGTCATACTT	GAAGCTAGAC
40	AGGCTTATCT	TGACAAGAA	GAAGATCGCT	TGGCCTCGCG	CGCAGATCAG
	TTGGAAGAAT	TTGTCCACTA	CGTGAAAGGC	GAGATCACCA	AGGTAGTCGG
	CAAATAAATA	ACTTCGTATA	ATGTATGCTA	TACGAAGTTA	Ttagctagc

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35 While certain of the preferred embodiments of the
present invention have been described and specifically
exemplified above, it is not intended that the invention
be limited to such embodiments. Various modifications
may be made thereto without departing from the scope and
spirit of the present invention, as set forth in the
following claims.

What is claimed is:

1. A site specific recombination method for
5 removal of predetermined nucleic acid sequences from the
plastid genome, said method comprising:

a) providing a first nucleic acid construct,
said construct comprising a promoter being operably
linked to a nucleic acid encoding an optional plastid
10 targeting transit sequence which is operably linked to a
nucleic acid encoding a protein having excision
activity, said construct further comprising a first
selectable marker encoding nucleic acid having plant
specific 5' and 3' regulatory nucleic acid sequences;

15 b) providing a second DNA construct, said
second construct comprising an second selectable marker
encoding nucleic acid and excision sites, said second
construct optionally containing a gene of interest, said
second construct further comprising flanking plastid
20 targeting nucleic acid sequences which facilitate
homologous recombination into said plastid genome;

c) introducing said second DNA construct into
a plant cell;

25 d) culturing said plant cell of step c) in the
presence of a selection agent, thereby selecting for
those plant cells expressing the proteins encoded by
said second DNA construct;

30 e) introducing said first DNA construct into
plant cells from step d) in the presence of a selection
agent and selecting those plant cells expressing
proteins encoded by said first construct, which when
present said excising activity acts on said excision
sites, thereby excising said predetermined target
sequence.

35

2. A method as claimed in claim 1, wherein a plant is regenerated from plant cells of step c), cells are then contacted with said first construct and steps d) and e) are performed.

3. A method as claimed in claim 1, wherein said first construct is that depicted in Figure 3.

4. A method as claimed in claim 1, wherein said second construct is that depicted in Figure 2.

5. A method as claimed in claim 1, wherein said protein having excision activity is selected from the group consisting of CRE, flippase, resolvase, FLP, SSV1-encoded integrase, and transposase.

6. A method as claimed in claim 1, wherein said excision sites are LOX sequences, and *frt* sequences.

7. A method as claimed in claim 1, wherein said selection agent is selected from the group consisting of kanamycin, gentamycin, spectinomycin, streptomycin and hygromycin, phosphinotricin, basta, glyphosate and bromoxynil.

8. A method as claimed in claim 1, wherein said excision of said predetermined sequence creates an expressible translational fusion protein.

10. A method as claimed in claim 1, wherein said predetermined target sequence is the selectable marker encoding nucleic acid present in said second construct.

11. A plant regenerated from the method of claim 1.

12. A site specific recombination system comprising the constructs of claim 1.

13. A site specific recombination method for removal of predetermined nucleic acid sequences from the plastid genome, said method comprising:

a) providing a first nucleic acid construct, said construct comprising a regulated promoter being operably linked to a nucleic acid encoding an optional plastid targeting transit sequence which is operably linked to a nucleic acid encoding a protein having excision activity, said construct optionally further comprising a first selectable marker encoding nucleic acid having plant specific 5' and 3' regulatory nucleic acid sequences;

b) providing a second DNA construct, said second construct comprising an second selectable marker encoding nucleic acid and excision sites, said second construct further comprising flanking plastid targeting nucleic acid sequences which facilitate homologous recombination into said plastid genome at a predetermined target sequence such that excision sites flank said predetermined target sequence following homologous recombination;

c) introducing said second DNA construct into a plant cell;

d) culturing a plant cell of step c) in the presence of a selection agent, thereby selecting for those plant cells expressing the proteins encoded by said second DNA construct;

e) regenerating a plant from cells obtained in step d);

f) introducing said first DNA construct into plant cells from step e) in the presence of a selection agent and selecting those plant cells expressing

proteins encoded by said first construct, which when present said excising activity acts on said excision sites, thereby excising said predetermined target sequence.

5

14. A method as claimed in claim 13, wherein said regulatable promoter is selected from the group of promoters consisting of inducible promoters, tissue specific promoters, developmentally regulated promoters and chemically inducible promoters.

10

15. A method as claimed in claim 13, wherein said predetermined target sequence is selected from the group consisting of genes associated with male sterility, *clpP* ribosomal proteins, ribosomal RNA operon sequences.

15

16. A method as claimed in claim 13, wherein said protein having excision activity is selected from the group consisting of CRE, flippase, resolvase, FLP, SSV1-encoded integrase, and transposase.

20

17. A method as claimed in claim 13, wherein said excision sites are LOX sequences, and *frt* sequences.

25

18. A method as claimed in claim 13, wherein said selection agent is selected from the group consisting of kanamycin, gentamycin, spectinomycin, streptomycin and hygromycin, phosphinotricin, basta, glyphosate and bromoxynil.

30

19. A plant regenerated from the method of claim 13.

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20. A site specific recombination system for removal of predetermined nucleic acid sequences comprising the construct of claim 13.

5 21. Progeny plants obtained from the plant of claim 11.

22. Progeny plants obtained from the plant of claim 18.

10

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: SITE-SPECIFIC RECOMBINATION SYSTEM TO MANIPULATE THE PLASTID GENOME OF HIGHER PLANTS

(57) Abstract: A site specific recombination system and methods of use thereof are disclosed for manipulating the genome of higher plants.



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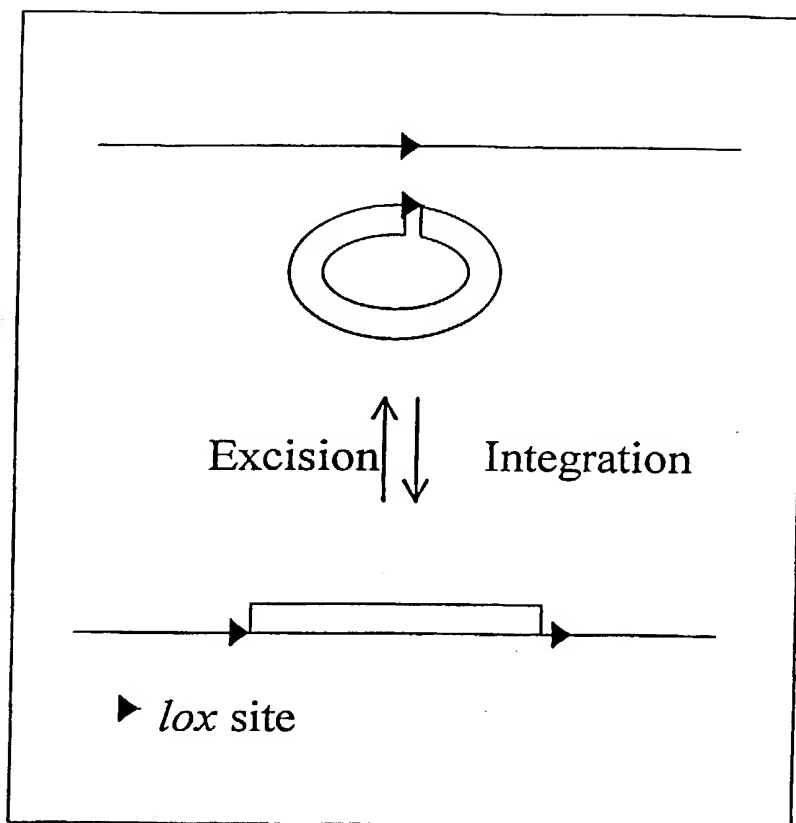


Figure 1



Figure 2

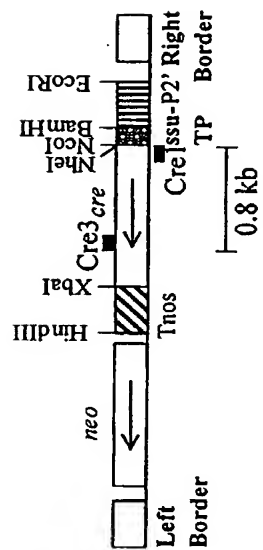


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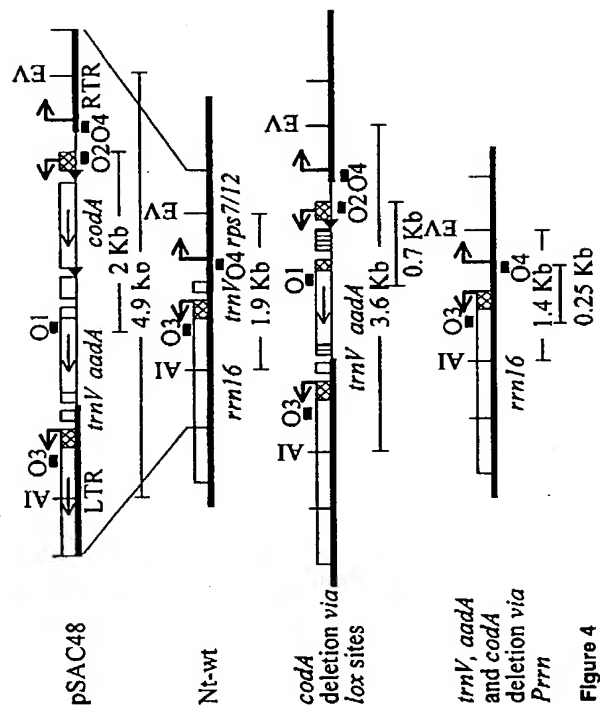


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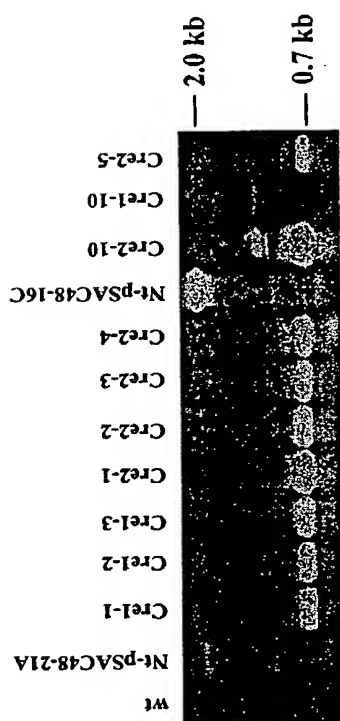


Figure 5

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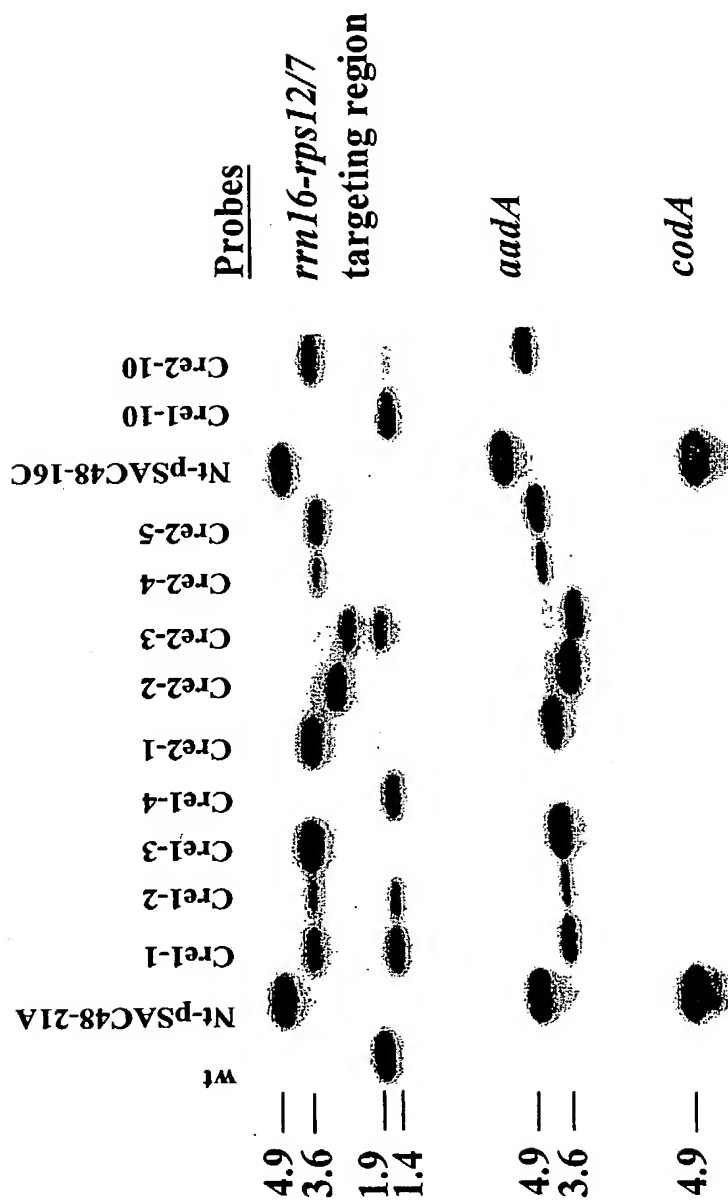


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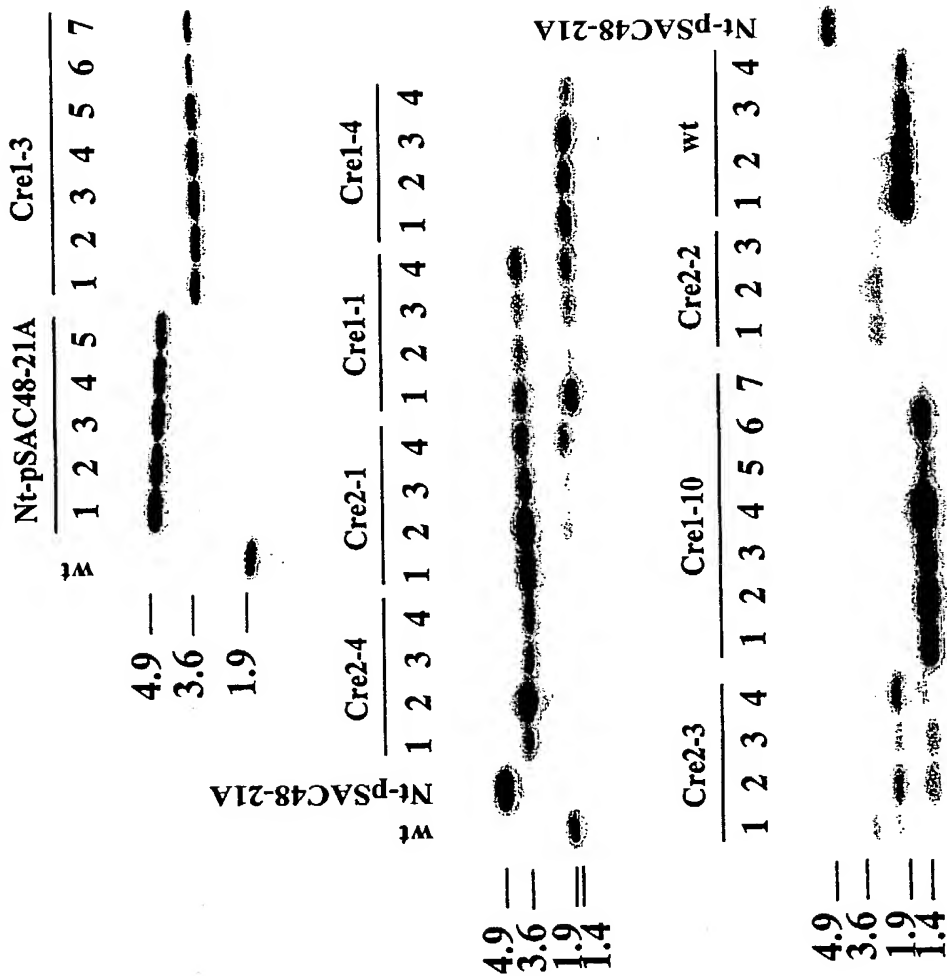


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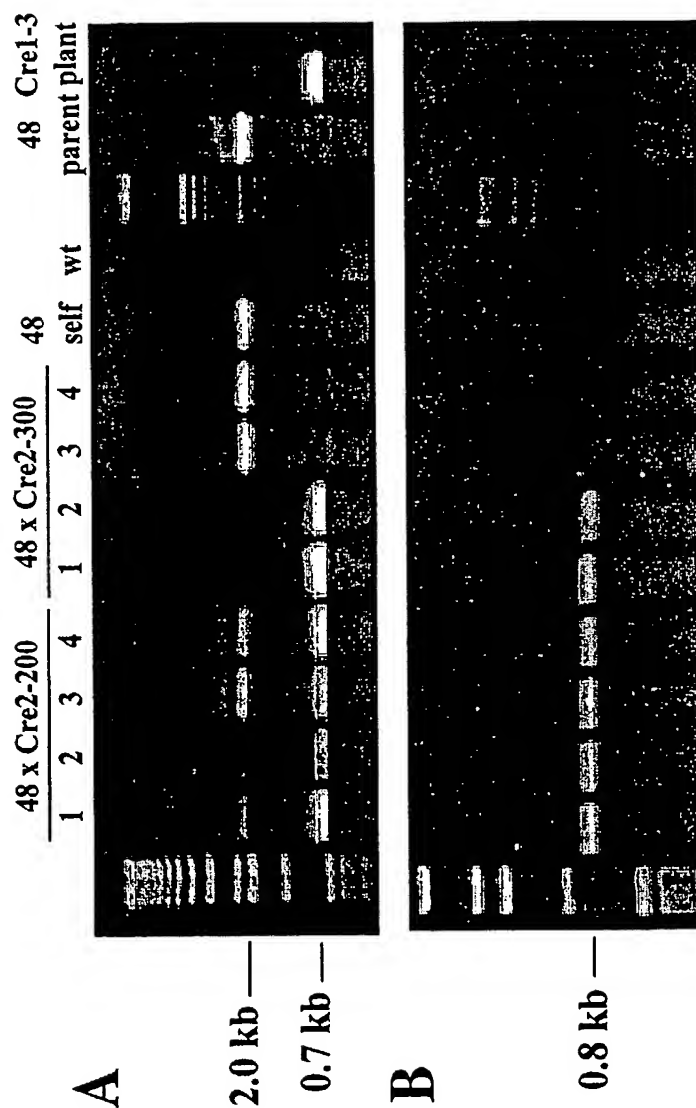
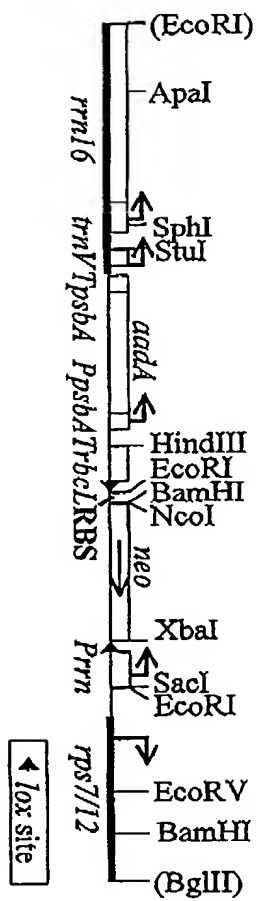


Figure 8

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Figure 9



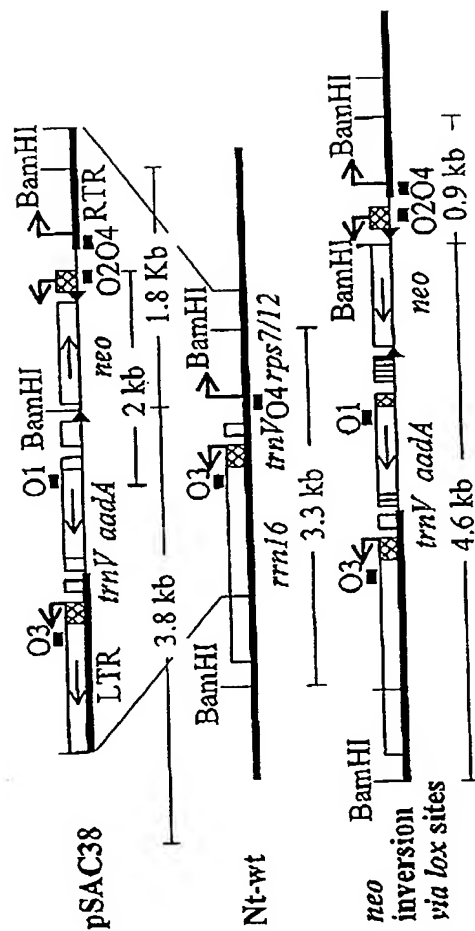


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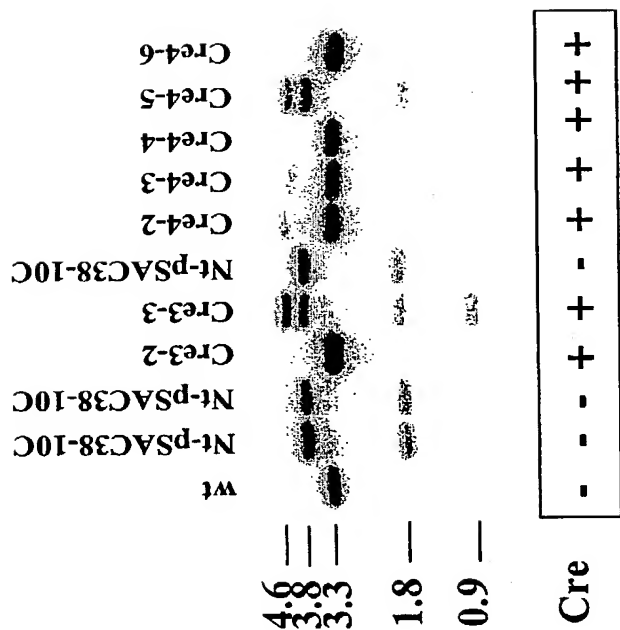


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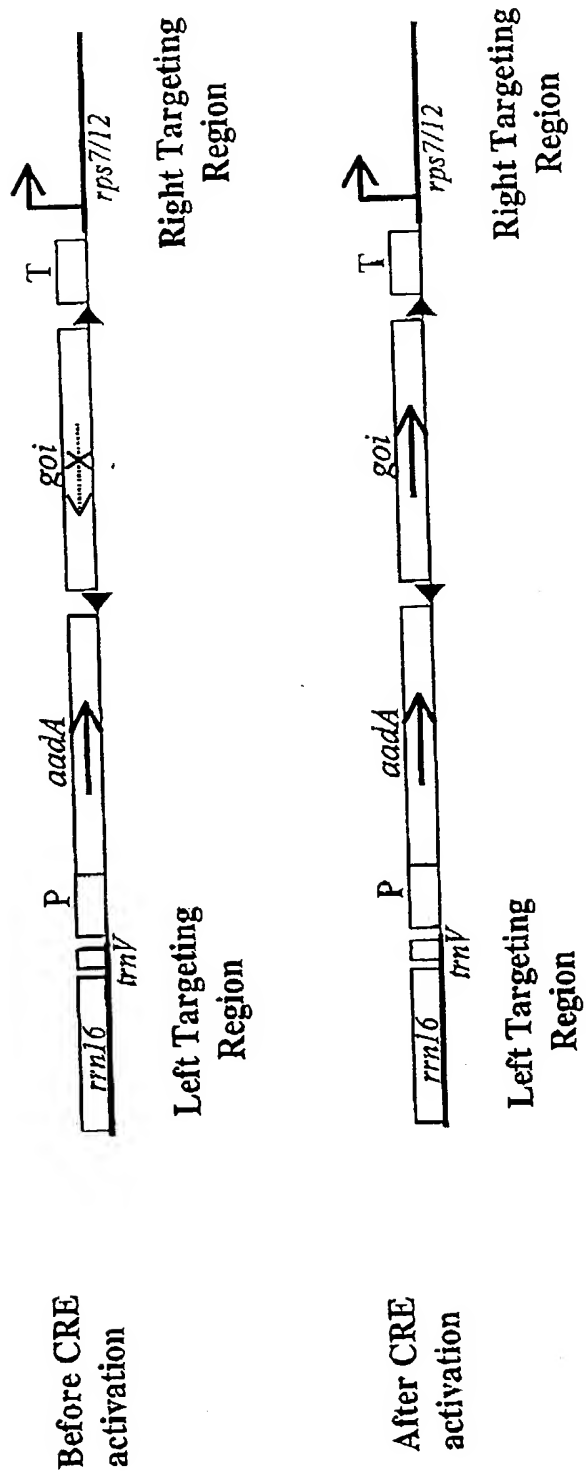


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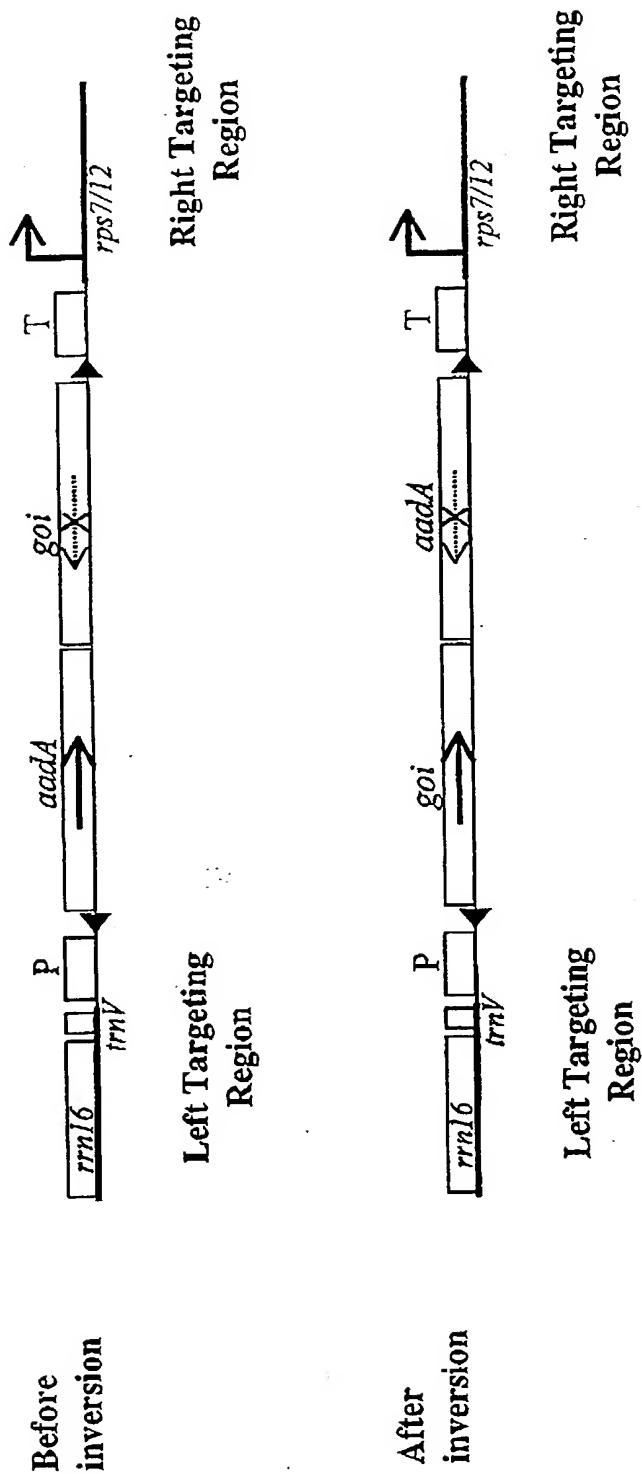


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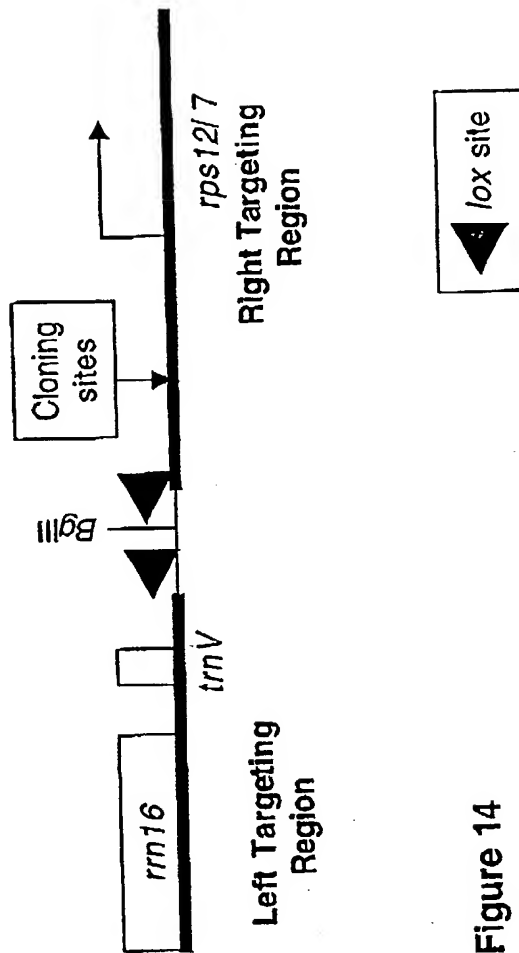


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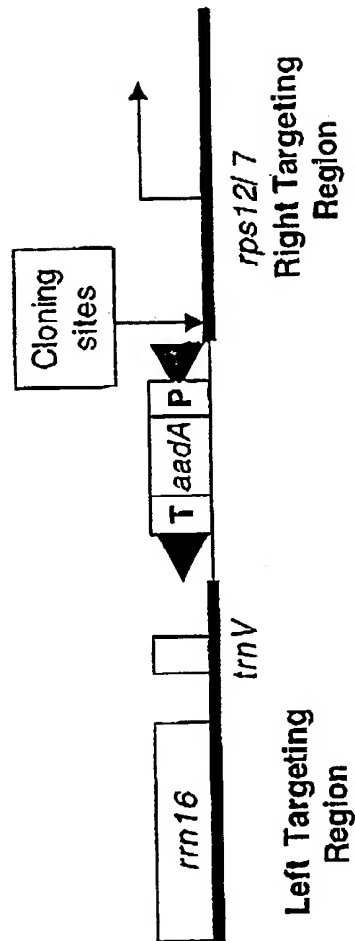


Figure 15

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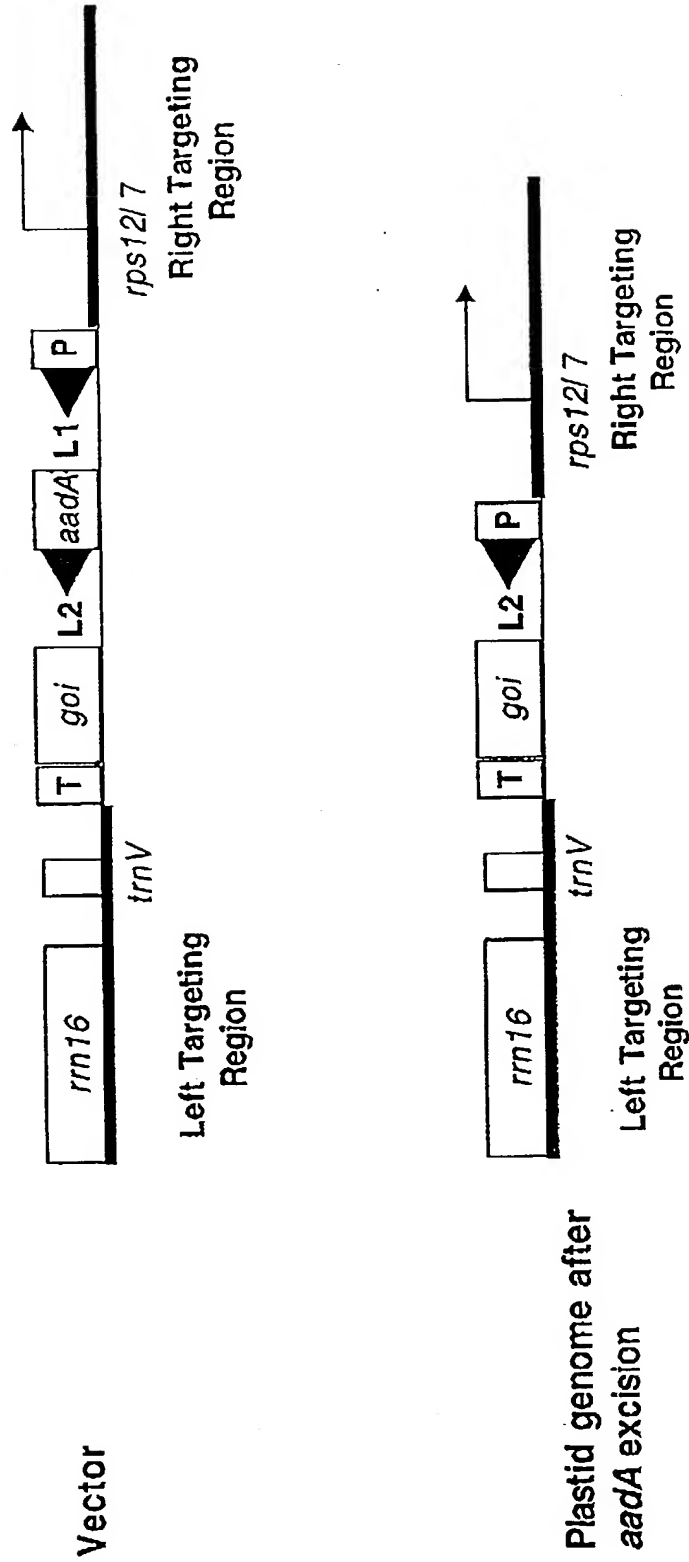


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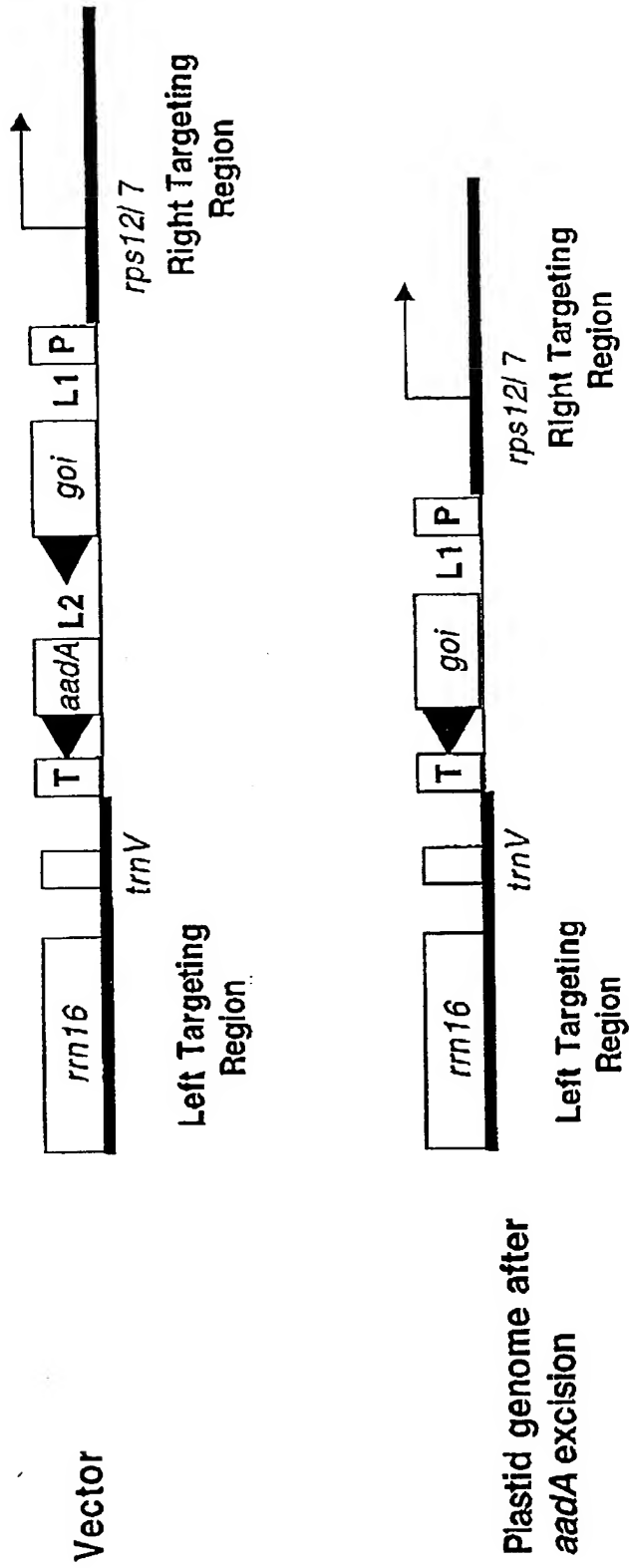


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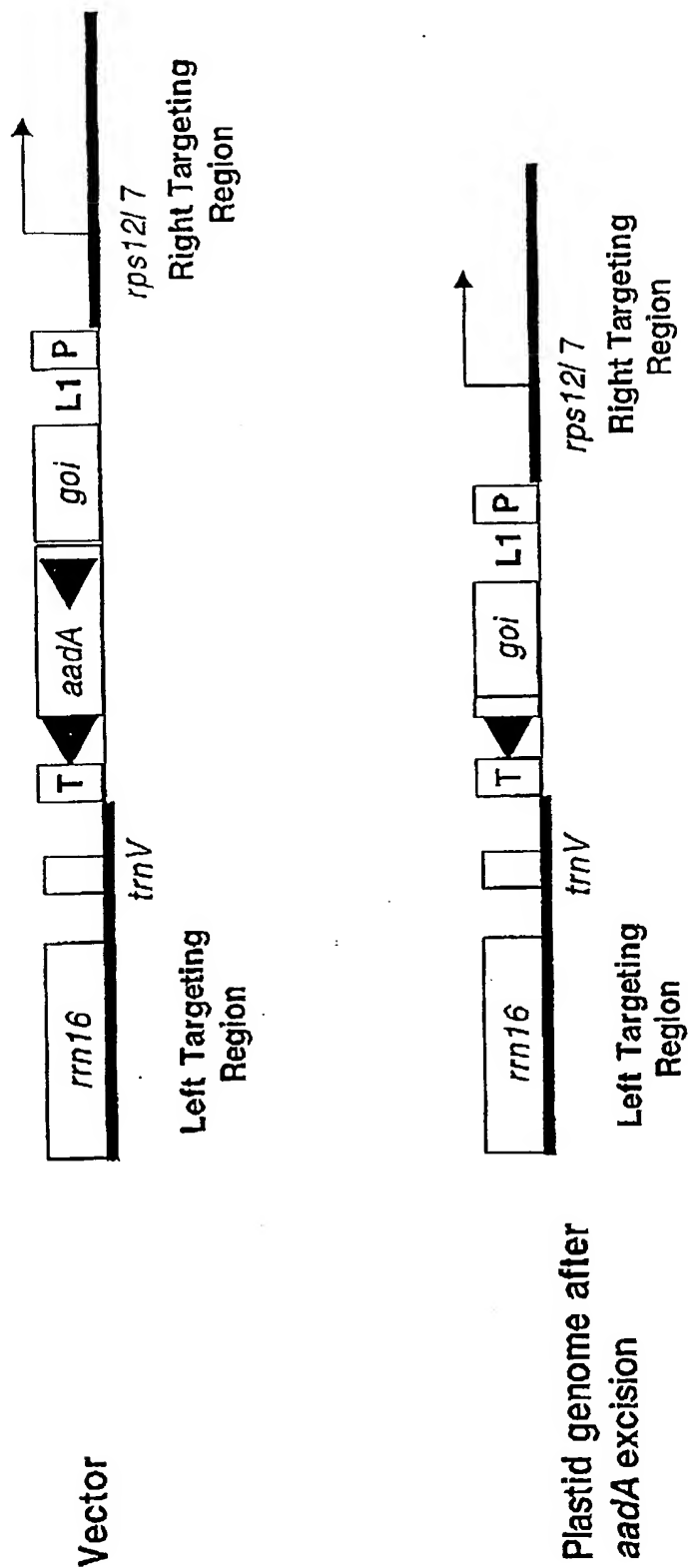


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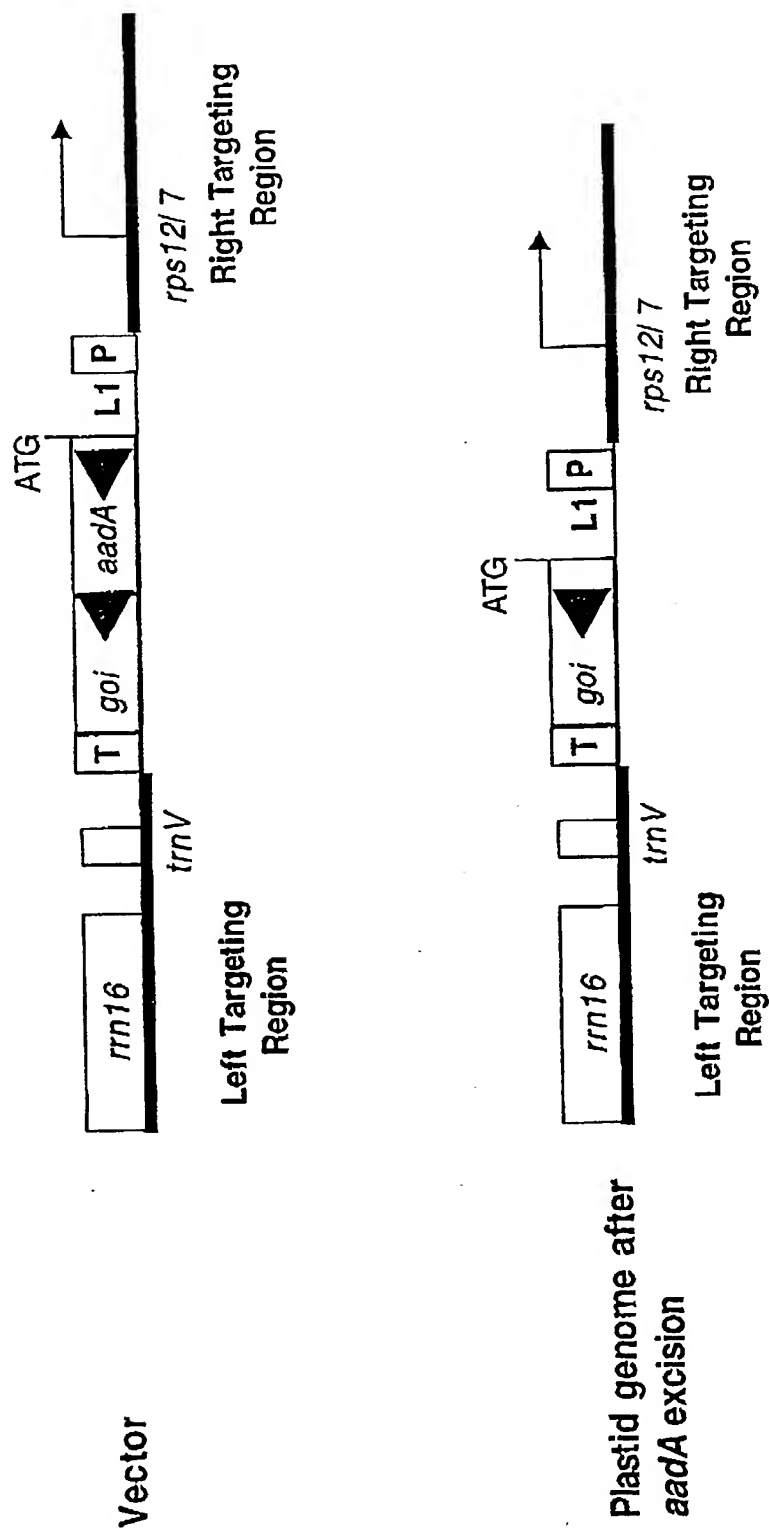


Figure 19

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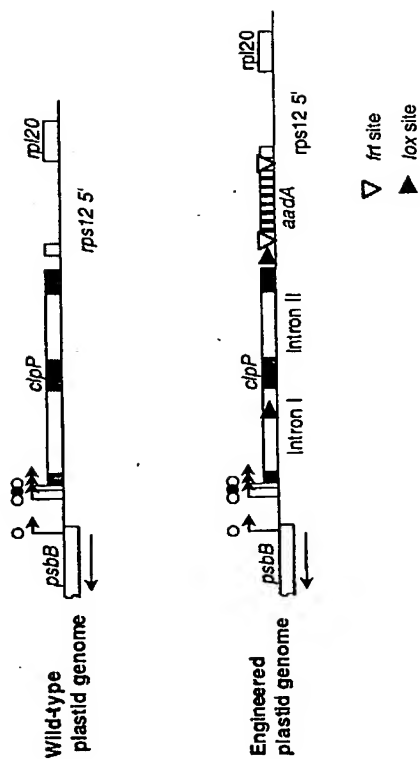


Figure 20



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DECLARATION, POWER OF ATTORNEY AND POWER TO INSPECT

As a below named inventor, I hereby declare:

that my residence, post office address and citizenship are as stated below next to my name;

that I verily believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural inventors are named below) of the invention entitled: **SITE-SPECIFIC RECOMBINATION SYSTEM TO MANIPULATE THE PLASTIC GENOME OF HIGHER PLANTS**

the specification of which (check one(s) applicable)

X was filed September 21, 2000 as U.S. International Patent Application No. PCT/US00/25930 on which U.S. Patent Application No. 10/088,634 file March 20, 2002 is based.
and was amended by Amendment filed _____ (if applicable); [or];
is attached to this Declaration, Power of Attorney and Power to Inspect;

that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above; and that I acknowledge my duty to disclose information which is material to the examination of this application in accordance with Rule 56 (a) [37 C.F.R. §1.56(a)].

CLAIM UNDER 35 USC §119(e): I hereby claim the benefit under 35 USC §119(e) of any United States provisional applications listed below:Provisional Application No.Filing Date
Day/Mo/Yr60/155,007
60/211,13021 September 1999
13 June 2000**POWER OF ATTORNEY:** As inventor, I hereby appoint **DANN, DORFMAN, HERRELL AND SKILLMAN, P.C.** of Philadelphia, PA, and the following individual(s) as my attorneys or agents with full power of substitution to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: **Kathleen D. Rigaut, Ph.D., J.D. Reg. No. 43,047; Patrick J. Hagan, Esq. Reg. No. 27,643 and Maria Kourtakis, Esq. Reg. No. 41,126****POWER TO INSPECT:** I hereby give **DANN, DORFMAN, HERRELL AND SKILLMAN, P.C.** of Philadelphia, PA or its duly accredited representatives power to inspect and obtain copies of the papers on file relating to this application.**SEND CORRESPONDENCE TO: CUSTOMER NUMBER 000110.****DIRECT INQUIRIES TO:** Telephone: (215) 563-4100
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U.S. Patent Application No. 10/088,634
Page 2 of Declaration, Power of Attorney and
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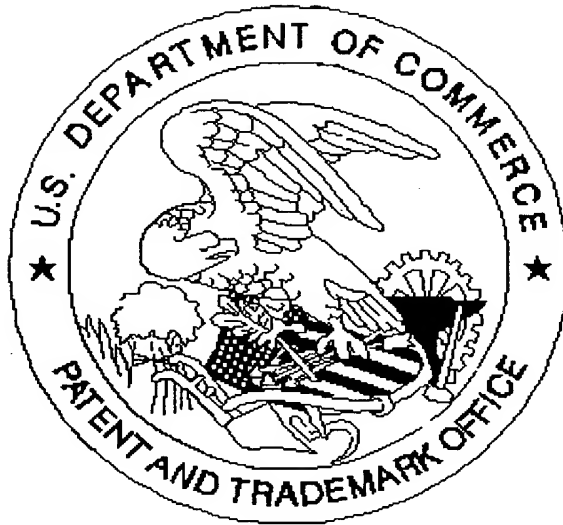
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